Transposon mutagenesis and characterisation of the selenite reduction pathway in *Methylosinus trichosporium*

By

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To my husband Azalden

Abstract

This study aimed to identify genes responsible for remediation of selenite pollution in the methane-oxidising bacterium *Ms. trichosporium* OB3b. It was proposed to achieve this by making a library of mutants inactivated in different genes and screening these mutants to see which were deficient in the remediation reactions and hence identify genes that are involved.

The pSAM_Rl plasmid, which contains the mariner transposon and was developed for use in the *Rhizobiaceae*, was introduced into *Ms. trichosporium* by conjugative transfer of the plasmid from *E. coli* SM10 *λpir*. The progeny from the conjugation were kanamycin (Km) resistant (strongly suggesting they contain the plasmid) and resistant to nalidixic acid (confirming removal of the *E. coli* donor strain). The presence of the transposon and its distribution around the chromosome were confirmed by genome sequencing of 18 randomly selected clones.

To make a transposon library suitable for screening, single colonies were picked from the conjugation plates onto a regular 6 × 8 grids on fresh plates to allow transfer of the library into 96-well plates with a Microplate Replicator. The individual clones of the library were stored in NMS medium with 30% glycerol at -80ºC in 96-well plates. 5,500 clones prepared in this way were screened on NMS agar with Km and selenite, to identify any mutants that were unable to reduce selenite to red elemental selenium. Screening of the library identified a mutant with liminished selenite-reduction activity. This mutant was inactivated in one of two gene copies of *pmoB*, encoding the largest subunit of the particulate methane monooxygenase involved in oxidizing methane to methanol. Hence, it may be impaired in selenite reduction due to a general deficiency of reducing equivalents within the cell.

Parallel experiments investigating the cellular localization of selenite-reducing activity indicated that the cytoplasmic selenite-reducing activity is likely to be due to a small molecule, because when the proteins of cytoplasm were digested the cytoplasm still reduced selenite.

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In preparation

Hania Aween, Alyaa Abdelhameed, Prachi Stafford and Thomas J. Smith. Development of a transposon mutagenesis system for *Methylosinus trichosporium* OB3b and Identification of genes that are involved in reduction of selenite.

Declaration

I hereby declare that the work presented in the thesis entitled “Transposon mutagenesis and characterisation of the selenite reduction pathway in *Methylosinus trichosporium*” in fulfilment of the requirements for the award of the Degree of Philosophy, Biomolecular Science Research Centre, Sheffield Hallam University, is an authentic record of my own work carried out under the supervision of Thomas J. Smith and Prachi Stafford. The matter embodied in this thesis has not been submitted in part or full to any other University or institute for the award of any degree in the UK or abroad.

Hania EL. Aween
March 2019

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List of abbreviations

Amp	Ampicillin
BCA	Bicinchoninic acid assay
BLAST	basic local alignment search tool
bp	base pair
BSA	Bovine serum albumin
Cr (III)	Chromium 3
Cr (VI)	Chromium 6
DNA	deoxyribonucleic acid
ENA	European Nucleotide Archive
FASTA	Federal Acquisition Streamlining Act
g	gram
h	Hours
INSeq	technique of insertion sequencing
Km	Kanamycin
LB	Luria-Bertani
MCM	Mauve Contig Mover
mg	milligram
ml	millilitre
mM	milli molar
NCBI	National Centre for Biotechnology Information
Ng	Microbes gene
ng	nanogram
NMS	nitrate mineral salts
OD	Optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pH	$-\log_{10}[H^+]$
ppm	part per million

pMMO	particulate methane monooxygenase
SDS	sodium dodecyl sulphate
Se	selenium
Se 0	elemental selenium
SeO_3^{2-}	selenite
SeO_4^{2-}	selenate
sMMO	soluble methane monooxygenase
TAE	tris acetate EDTA
TCE	trichloroethylene
TEs	transposable elements
UV	ultra violet
v/v	volume/volume
w/v	weight/volume
µl	microlitre
$kJmol^{-1}$	kilocalorie per mole

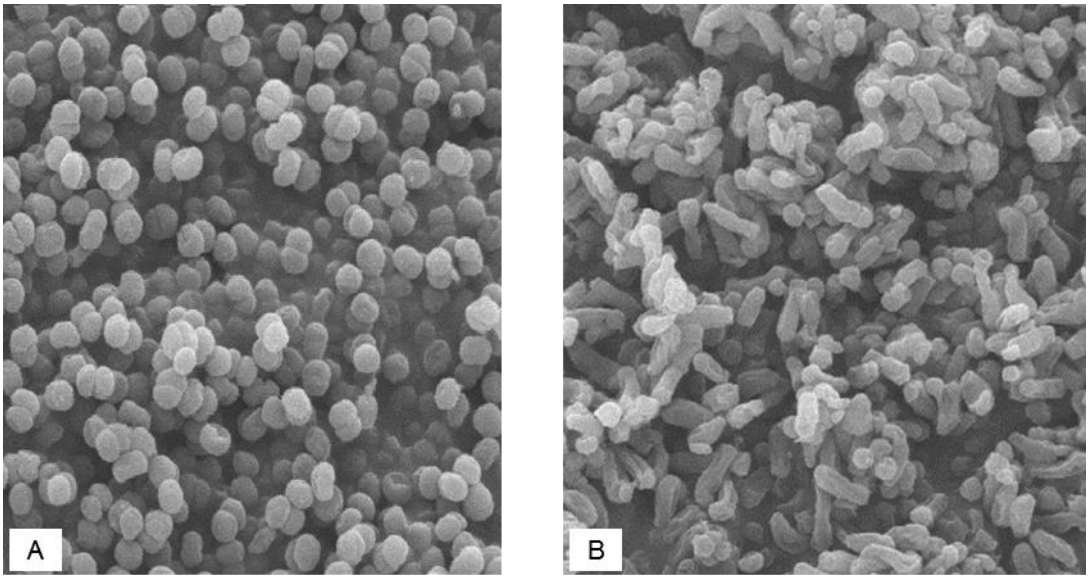
Chapter 1 Introduction

1.1. Literature Review

1.1.1. Methane-oxidising bacteria

Methane-oxidising bacteria (Methanotrophs) are a group of bacteria that can use methane as their sole source of carbon and energy (Hanson, Hanson, 1996; Semrau, DiSpirito & Yoon, 2010). Methane is one of the most difficult organic substrates to activate, with C-H bond dissociation energy of 435 kJmol. In biology, there are only two groups of enzymes that can perform this reaction. The first one is composed of the soluble methane monooxygenases, which contain a dinuclear Fe active site, similar to that of the ribonucleotide reductases. This is a well-characterised group of enzymes, and their reaction mechanism is understood in some detail. The second group contains the particulate membrane-bound) methane monooxygenases (pMMO), which are the predominant enzymes in methanotrophic bacteria, except under copper-limiting conditions (Cao *et al*., 2018).

Methane-oxidising bacteria are well known for their role in reducing methane (greenhouse gas) load in atmosphere (Smith, Murrell, 2009). Methanotrophs are aerobic and Gram-negative bacteria, they are widespread in the environment, including sediments, soils, water and muds. They exist in a variety of habitats due to having physiologically versatile nature and found in a wide range of pH, temperature, oxygen concentrations, salinity, heavy metal concentrations, and radiation (Pandey *et al*., 2014). Recently, it was observed that the methanotroph present in biogenic methane and natural gas seep environments, *Methylocella silvestris* grew on ethane and propane, other components of natural gas, in present of methane; it may play a significant role in biogeochemical cycling of gaseous hydrocarbons (Haque *et al*., 2018). Methanotrophs are able to oxidize a wide range of substances ranging from methane to naphthalene and biphenyl including very toxic materials (Dalton, 2005) (Fig 1.1 shows examples of methanotrophs).



Methylococcus capsulatus (Bath) (A) *Methylosinus trichosporium* OB3b (B)

Figure 1-1. Examples of methanotrophs

(Abdurrahman Eswayah, personal communication)

1.1.2. Taxonomy of methanotrophic bacteria

Methanotrophs have been known for more than 100 years. Sohngen was the first to characterise a bacterium from aquatic plants and from pond water; the bacterium was able to grow on methane. It was named *Bacillus methanicus*. Unfortunately, this species became lost. However, many attempts were made subsequently to isolate methane-oxidizing bacteria. Foster's laboratory began to isolate methanotrophic bacteria from different sources. Bacteria named *Pseudomonas methanica* for Sohngen's*B. Methanicus*when, and then isolated them again; additionally, they isolated a methane oxidizer belonging to a new genus, *Methylococcus capsulatus* (Foster, Davis, 1966).

However, these attempts were not successful due to the difficulty involved in separating the methanotrophs from contaminating heterotrophic bacteria. Whittenbury, *et al*., (1970) were successful in obtaining colonies of methanotrophic bacteria without the presence of other bacteria by using plate microscopy to identify tiny colonies on plates uncontaminated by heterotrophic organisms. There are now more than one hundred known species of aerobic bacterium that can use methane as their only source of carbon and energy.

Whittenbury *et al*., (1970), also provided the scientific community with the first detailed study on the isolation, classification and physiology of methanotrophs. These researchers classified them on the basis of their morphological features and ultra-structure of cells. These bacteria were assigned to five genera, which were categorized into two types. Type 1 methanotrophs have intracytoplasmic membranes, which form disc like shaped vesicles in the cells. Type I contains three genera of methanotrophs; *Methylococcus, Methylobacter* and *Methylomonas.* Type II methanotrophs comprises *Methylosinus* and *Methylocystis* and it is characterized by intracytoplasmic membranes organized on the periphery of the cells.

The number of genera and species of methanotrophs has recently increased because new techniques have been applied to the analysis of methanotrophs and their isolation from the environment. The majority of methanotraphs contains particulate methane monooxygenase enzyme as shown in Table 1.1. A small number of methanotrophs only possesses the soluble methane monooxygenase enzyme as displayed in Table 1.2 & Table 1.3 demonstrates some of the methanotrophic species that can produce both particulate soluble and methane monooxygenase enzymes (Theisen *et al*., 2005).

Table 1-1. Methanotrophs that contain pMMO

Genus	Phylogenic group	References
Methylobacter	*Gammaproteobacteria*	(Foster and Davis 1966)
Methylocaldum		(Bodrossy *et al.*, 1997)
Methylohalobus		(Heyer *et al*., 2005)
Methylomicrobium		(Bowman *et al*., 1995)
Methyomonas		(Leadbetter *et al*., 1974)
Methyosoma		(Rahalkar *et al*., 2007)
Methyosphaera		(Bowman *et al*., 1997)
Methyosarcina		(Wise *et al*., 2001)
Methylothermus		(Tsubota *et al.*, 2005)
Methylogaea		(Geymonat *et al*., 2011)
Crenothrix		(Stoecker *et al*., 2006)
Clonothrix		(Vigliotta *et al*., 2007)
Methylacidiphilum	*Verrucomicrobia*	(Pol *et al*., 2007; Dunfield *et al.*, 2007)

Table 1-2. Methanotrophs that contain sMMO

Genus	Phylognetic group	References
Methylocapsa	*Alphaproteodacteria*	(Dedysh *et al*., 2002)
Methylocella		(Dedysh *et al*., 2000)
Methyloferula		(Vorobev *et al.*, 2011)

Table 1-3**.** Methanotrophs that contain pMMO and sMMO

Genus	Phylognetic group	References
Methylococcus	*Gammaproteobacteria*	(Foster and Davis 1966)
Methylocystis	*Alphaproteobacteria*	(Whittenbury, *et al*.,1970)
Methylosinus		(Whittenbury, *et al.*,1970)
Methylovulum		(Iguchi, *et al*., 2011)
Methylorosula		(Berestovskaya *et al.*, 2012)

1.1.3. Methane monooxygenase (MMO)

Methane-oxidizing bacteria have a unique ability to use methane for growth by a process in which methane is oxidized by the enzyme, methane monooxygenase (MMO) (Dalton, 2005) (Equation 1.1).

$$CH_4 + NAD(P)H + H^+ + O_2 \xrightarrow{MMO} CH_3OH + NAD(P)^+ + H_2O$$

Equation 1.1. MMO catalyzes a classic monooxygenase reaction in which two reducing equivalents from NAD(P)H are utilized to split the O-O bond of O_2. One atom of oxygen is reduced to water while the second is incorporated into the substrate to yield methanol (Dalton, 1980)

As indicated above two types of MMO are known; particulate methane monooxygenase (pMMO) and soluble methane monooxygenases (sMMO). Both are multicomponent enzymes (Murrell *et al.,* 2000). The well-studied methanotrophs *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* produce the two forms of MMOs (Dalton, 2005) (Table 1-3). Methanotrophs such as *Methylomicrobium album* BG8 and *Methylomona smethanica*, produce only pMMO (Hanson, Hanson, 1996).

Production of sMMO and pMMO in the cell depends on the amount of copper, when the ratio of copper to biomass is very low; sMMO is elaborated rather than the pMMO which expressed at a high copper to biomass ratio (Stanley *et al.,* 1983). It is found out that a relationship exists between the amount of copper in the medium and the activity of pMMO. The activity of pMMO is increased by the presence of copper (Nguyen *et al.*, 1994: Stanley *et al.,* 1983). DNA sequencing studies showed that pMMO is encoded by three genes *pmoC*, *pmoA*, and *pmoB* organised as an operon *pmoCAB*, of which there are multiple copies in the genome of many methanotrophs. For example, the genome of *Ms. trichosporium* OB3b has two copies of *pmoCAB* (Matsen et al., 2013; Gilbert et al., 2000). The genes *pmoC*, *pmoA*, and *pmoB* encode

for pMMO γ (22 kDa), β (24 kDa), and α (45 kDa) subunits respectively (Semrau *et al.*, 1995).

Despite that both pMMO and sMMO can use methane and convert it to methanol, there is a large difference in the amino acid sequences of their protein components. A pMMO is membrane-associated whereas sMMO is soluble; it is located in the cytoplasm (Dalton, 2005). The conversion of methane (which has a very unreactive C-H bond) to methanol chemically needs a high energy > 435 kJ mol^{-1}, hence the conversion of methane to methanol by methane monooxygenase makes this enzyme potentially interesting commercially (Rosenzweig *et al*., 1997).

Many studies showed that when some methanotrophs grow in a narrow range of oxygen and a high level of nitrite conditions, pMMO will be expressed, while sMMO is produced in the opposite conditions. However, to distinguish between pMMO and sMMO by a colorimetric rapid, the naphthalene test (Brusseau *et al.,* 1990) can be used. This can prove the production of sMMO by the bacteria, the unique enzyme that can oxidize it as a substrate while pMMO cannot (Smith, Dalton, 2004). To characterize pMMO in a membrane-like environment, Ro'I *et al.,* (2018) reconstituted pMMOs from *Methylomicrobium (Mm.) alcaliphilum* 20Z and *Methylococcus (Mcc.) capsulatus* (Bath) into bicelles. Reconstitution into bicelles recovers methane oxidation activity lost upon detergent solubilization and purification without substantial alterations to copper content or copper electronic structure as observed by electron paramagnetic resonance (EPR) spectroscopy.

1.1.4. Remediation by methanotrophs

De Marco (2004) and his colleagues attempted to systematically analyse the ability of methylotrophic strains to improve tolerance against heavy metal pollutants. These workers isolated thirty-one novel methylotrophic bacterial strains from a range of sediment and soil sources. Moreover, they noted some of the isolates exhibited interesting characteristics of resistance to arsenate, heavy metals, or organic pollutants. Four strains from this work were

considered as real ‘super-bugs’ for their ability to withstand extremely high concentrations of a diversity of heavy metal pollutants. Boden, Murrell (2011) discovered that *Methylococcus capsulatus* (Bath) is capable of reducing highly toxic mercury (II) ions to less toxic elemental mercury. It has since been shown that *Mc. capsulatus* (Bath), like other bacteria, uses an NAD (P) H-dependent mercuric reductase to detoxify mercury (Pandey *et al*., 2014).

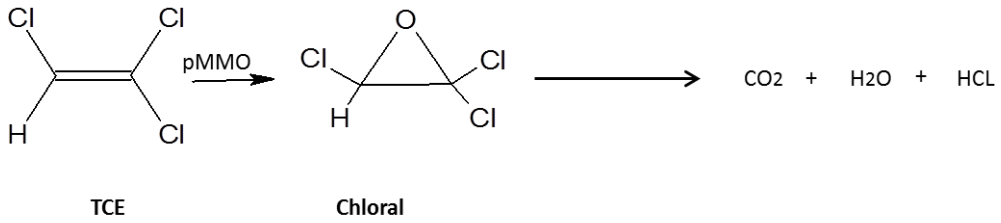
Most of the work that has been done to develop methanotrophs for remediation organic pollutants has centred on the ability of the MMO enzymes to oxidise a range of hydrophobic organic molecules. pMMO has fewer substrates compared to sMMO. The substrates of pMMO are methane, and straight short-chain hydrocarbons. It does not oxidise aromatics (e.g. benzene, ethyl benzene and styrene), the branched aliphatic 2-methylpropane or alicyclic C-H compounds such asCyclohexane (Burrows *et al*., 1984), though all these compounds are sMMO substrates. It appears that the entry to the active site of pMMO is more restricted than that to sMMO. Acetylene is a strong suicide substrate for pMMO and sMMO (Prior, Dalton, 1985), whereas phenylacetylene has a greater effect on sMMO than on pMMO (Lontoh *et al*., 2000).

Both pMMO and sMMO can oxidise the pollutant trichloroethene. With sMMO, rates comparable to the oxidation of methane were observed; whilst with pMMO the rate of trichloroethene breakdown was low. However, the detected products of trichloroethene breakdown by pMMO expressing methanotrophs do not include chloral, a pollutant that may be more harmful than trichloroethene which has been detected in sMMO-mediated trichloroethene breakdown. Hence bioremediation of trichloroethene by pMMO may have some advantages. During trials of methane-enhanced bioremediation of groundwater, both sMMO and pMMO genes were detected and it is likely that both were expressed to some extent in the population of methanotrophs present. (Smith, Dalton, 2004).

Trichloroethylene (TCE) and chloroform, which occur as halogenated hydrocarbon contaminants of groundwater, cannot be used by methanotrophic bacteria as a source of carbon and energy (Hanson, Hanson, 1996) although

methanotroph cells or purified pMMO can transform and mineralise TCE (Lontoh *et al.*, 2000).

Complete degradation of TCE can be performed by a methane-oxidising consortium of bacteria as in Equation 1.2, in which methanotrophs and other types of bacteria may perform different steps in the complete oxidation of TCE (Alvarez-Cohen, McCarty, 1991; Chang, Alvarez-Cohen 1995).



Equation 1.2 Degradation of TCE

An alternative to bioremediation of TCE using a methanotroph culture is to alternate between bioremediation of TCE, where the methanotroph is progressively inhibited by the pollutant, and growth on methane where the culture can recover. This was trialled in a sequencing biofilm reactor by (Speitel, Leonard, 1992).

Yoon *et al*., (2011) described a facultative methanotroph *Methylocystis* strain SB2 that is capable of oxidizing chlorinated ethanes. Im, Semrau (2011) showed that *Methylocystis* strain SB2 can oxidize a wide range of chlorinated hydrocarbons, such astrans-dichloroethylene (t-DCE), vinyl chloride (VC), TCE, 1,1,1,-trichloroethene (1,1,1,TCA) and chloroform (CF), when growing on methane. It also oxidized VC, t-DCE, TCE, and 1, 1, 1, TCA rather than CF when ethanol was used for growth. The degradation of these substances was inhibited when acetylene was added, which confirmed that sMMO and pMMO were responsible for the degradation. These researchers also proposed that when facultative methanotrophs grow on alternative substrates, they make them concentrate on pollutants oxidation which is an attractive property for use in bioremediation.

Chromium VI is a highly oxidizing, soluble, mutagenic, and detrimental metal species that is used in paper manufacturing, lamination, tanning, among other uses (Cervantes *et al.*, 2001). Al-Hasin *et al.* (2009) demonstrated that the well characterized methanotrophic bacterium *Mc. capsultatus* (Bath) is capable of transforming chromium VI at different concentrations reducing it to be less toxic chromium III. Chromium III has low bioavailability and is readily absorbed to negatively charged biopolymers and soil particles (Cervantes *et al*., 2001; Cheung *et al.*, 2007).

1.1.4.1. Reduction of selenite.

Selenium was discovered in 1817. It belongs to Group VIA of the Periodic Table and it has been classified as a metalloid. It has contrasting properties: metal and nonmetal (Crystal, 1973). Selenium (Se) is naturally occurring and exists in different oxidation states (-2, selenide; 0, elemental selenium; +4, selenite and +6, selenite) as in Fig (1.2). It has many chemical forms (inorganic and organic). Se is both essential and toxic because it is needed in small amounts for growth and metabolism in most living organisms; it is toxic at high concentration. Se occurs in terms of two soluble species: selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}). They are the most common Se oxyanions are found in soil solution and natural waters. Both oxyanions are of significant concern because they are toxic; they are identified to be accumulative toxins (Dungan, Frankenberger, 1999).

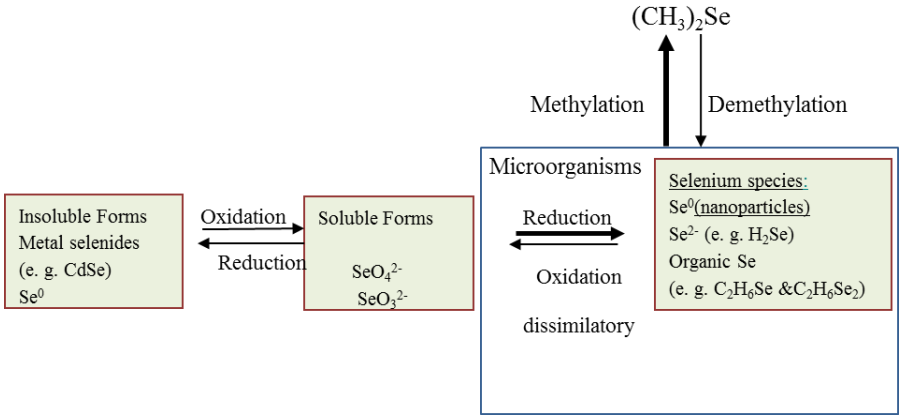


Figure 1-2.Schematic Se cycle in soil, and the influence of microbial processes on the transformation of the element.

The bold arrows indicate the preferred direction of the process (Eswayah *et al*. 2016).

On the other hand, many organisms have been reported to have the ability to transform these toxic forms of Se to less toxic or nontoxic forms by reduction, methylation, oxidation and demethylation as in Fig 1.2.

This encourages the idea of using these microorganisms to remediate Se-contaminated zones. Eswayah *et al.,* (2017) found that the methanotrophic bacteria *Ms. capsulatus* (Bath) and *Ms. trichosporium* OB3b were able to reduce the toxic SeO_3^{2-} to less / nontoxic elemental selenium. Also, they observed that the cultures produced volatile selenium-containing species, which suggests that these strains may have an additional activity that is able to transform Se^0 or selenite into volatile methylated forms of selenium. The results from experiments with the cell fractions cell wall, cytoplasm, cell membrane and transmission electron microscopy (TEM) measurements indicated that the nanoparticles are formed mainly on the cell wall with sizes less than 100 nm, as shown in Fig 1.2. In order to use microorganisms for Se remediation, a number of studies have been carried out in small and large-scale remediation schemes. Soda *et al.,* (2012) observed reduction of 95% of the soluble element when they treated Se-oxyanion-containing oil refinery wastewater using the SeO_4^{2-} respiring bacterium *thaueraselenatis*. Fujita *et al.* (2002) tested the ability of the SeO_4^{2-} reducing bacterium, *Bacillus* sp. strain SF-1, to remove SeO_4^{2} from a model wastewater containing 41.8 mg/l SeO_4^{2} under steady-state conditions with lactate as the electron donor and they observed that as the retention time was increased, more of the selenium was reduced to Se^0. Conversion of Se^0 was 99% at a cell retention time of 92.5 h and Se^0 production rate of 0.45 mg/ litre / h.

Hunter (2014a) evidenced that *Saccharomyces cerevisiae* glutathione reductase (GR) and *E. coli* thioredoxin reductase (ThxR) may be responsible for the reduction of SeO_3^{2-} to Se^0, proposing that *Pseudomonas seleniipraecipitans* GR and ThxR can also reduce SeO_3^{2-} to Se^0. (Hunter, 2014b) found a protein present in the cell-free extracts of *Rhizobium selenitireducens* that belongs to old-yellow (OYE) protein family. The enzyme reduces SeO_3^{2-} to Se^0 by using NADH as electron donor. Li *et al.* (2014) used Shewanella oneidensis MR-1 to study the contribution of anaerobic respiration system in the reduction of SeO_3^{2-} and recognized that fumaratere ductase

FccA as the terminal reductase of SeO_3^{2-} in the periplasm and found that the reduction is dependent on central respiration c-type cytochrome CymA.

Also, it has been reported that some algal species can volatilize substantial amounts of inorganic Se compounds (Neumann *et al*., 2003), so algal methylation of selenium species can be a possible method to remove selenium from the aqueous phase. Huang, Zeng & Zhang (2013) reported that the alga *Chlorella vulgaris* removed 96% of Se supplied as selenium oxyanions (1.58 mg liter1) from a microcosm water column after 72 h.

1.1.4.2 The physiology of selenite reduction reaction

Certain microorganisms can grow anaerobically through the dissimilatory reduction of selenium oxyanions. Kessi *et al.* (1999) reported that phototrophic bacteria such as *Rhodospirillum rubrum* are able to reduce selenite, which they detoxify to elemental selenium. They observed, in the culture medium after selenite reduction, a large amount of selenium-containing particles, which also suggested that *R. rubrum* is able to efficiently transport elemental selenium out of the cell. On the other hand, Electron micrographs (EM) were produced showing the cells were grown with 1.0 mM selenite and sampled after and during the reduction phase. Visibly, during the reduction, electron-dense particles were present more than after reduction completed.

Several studies have investigated the mechanisms of microbial formation of Se nanoparticles, where it has been found that Se nanoparticles are associated with proteins (Jain *et al*., 2015b; Song *et al*., 2017; Li *et al.,* 2014). *Shewanella oneidensis* MR-1 has fumarate reductase FccA as the terminal reductase of selenite in periplasm. Here, there is an organic layer covering the colloidal biogenic elemental selenium nanoparticles (BioSeNPs), which comprises extracellular polymeric substances (EPS) derived from the cells (Jain *et al*., 2015).

Some of bacterial species can only reduce SeO_3^{2-} (but not SeO_4^{2-}) to Se^0 (Bebien *et al*., 2001; Roux *et al*., 2001), for example, *Bacillus selenitireducens* MLS10 respiration via reduction of SeO_3^{2-} to Se^0 (Switzer Blum *et al*., 1998). The reduction of SeO_4^{2-} to Se^0 is usually a two-step process in which SeO_3^{2-}

is an intermediate product (Lortie *et al*., 1992), such as in *Pseudomonas stutzeri* NT-1, which performs aerobic reduction of SeO_4^{2-} and SeO_3^{2-} to Se^0 (Kuroda *et al*., 2011).

Eswayah *et al.* (2017) reported that *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b are able to reduce selenite to elemental selenium (S_0) and confirmed this observation by electron micrographs with corresponding EDXS spectra. They observed that the nanoparticles were spherical and in a variety of sizes associated with cells (in the extracellular space). The mean particle sizes in the *Ms. trichosporium* OB3b cultures was 221 nm compared to 387 nm for *Mc. capsulatus* cultures after 288 h and 48 L incubation, respectively (Fig 1.3). The EDXS analysis showed that the electron-dense particles contained selenium. Also, a stronger red colour was observed in the cultures of *Mc. capsulatus* with the larger elemental selenium particles in contrast to the yellowish orange observed in the cultures of *Ms. trichosporium* OB3b.

Reduction of selenite by the cell wall fractions occurred in the absence of methane. Also, since the sMMO-deleted mutant of *Ms. trichosporium* OB3b formed nanoparticles indistinguishable from the wildtype strain, it appears that the components of the sMMO enzyme system (including its NAD(P)H-dependent reductase) are not essential for the reduction of selenite. Since the cell wall fraction of the cells is capable of reducing selenite in the absence of added reducing agents, although the cultures as a whole require methane to perform the reaction, it seems that methane (activated either by sMMO or the particulate methane monooxygenase system) is likely the ultimate source of reducing agents, though other mediator(s) are involved in transferring the electrons to selenite. This opens the possibility that methanotrophs (which are widespread across diverse environments) may play a significant role in the global selenium cycle. The results also suggest that these bacteria may be useful in preparing selenium nanoparticles of a range of sizes for biotechnological applications. Much remains to be determined about the pathway of selenium biotransformations in methanotrophs, though it appears that elemental selenium may not necessarily be an intermediate on the pathway to the formation of all volatile selenium species. (Smith, Murrell, 2011; Eswayah *et al.*, 2016)

Figure 1. 3. TEM of *Mc. Capsulatus*, (a) and *Ms. trichosporium* OB3b (b) cultures exposed to SeO_3^{2-} (20 mg L^{-1}) and EDXS analysis in the electron-dense regions (Se^0 nanospheres)

1.1.4.2. Reduction of chromium

Chromium (VI) pollution in the environment is a global concern, and it may be present in all the phases of the environment such as air, soil and water (Tandon, Vikram, 2014). Chromium is a transition metal located in group VI-B of the periodic table. It is a highly toxic in the hexavalent form and non-essential metal for plants and microorganisms (Cervantes *et al*., 2001; Singh, Misra & Tandon 2006). It can also be found in oxidation states from -2 to +6,

but in the environment, chromium is present primarily in the stable oxidation states: +3 and +6 (Barnhart, 1997). Additional details on the comparison between chromium (VI) and (III) are given in Table 1.4. Chromium (VI) is known as a strong oxidizing agent, and it can be reduced to Cr (III) in the presence of organic matter as in Equation1.3.

$$CrO_4^{2-} + 3^e + 8H \longrightarrow Cr^{3+} + 4H_2O$$

Equation1.3 Chromium (VI) reduced to Cr (III)

In acid environments such as acidic soils, this transformation is faster. Moreover, chromium (III) can be oxidized to Cr (VI) in the presence of a high concentration of oxygen (Cervantes *et al*., 2001). As mentioned above, Al Hasin *et al* (2009) reported that *Mc. capsulatus* (Bath) can reduce chromium (VI) to chromium (III). The reduction reaction of chromium (VI) was based on the availability of reduction equivalents from the substrate growth methane and it was partially inhibited by the metabolic poison sodium azide. Also a chromate (VI) reducing activity has been identified in the methanotrophic bacterium *Metholococcus capsulatus* (Bath) that was able to reduce chromium (VI) to chromium (III), but the *Methylosinus trichosporium* OB3b did not detectably reduce the chromium (VI).

Chromium (Cr) can be both toxic and beneficial to humans and animals, it depends on the concentration of the Cr. They required a trace of amount for maintaining health (Katz, Salem, 1994)

Chromium (Cr) has great economic importance as industrial metal (Cervantes *et al*., 2001). The widespread usage of this element in industry has caused Cr to be a serious environmental pollutant of air, soil and water (Herna *et al.,* 1995). The presence of this metal in the environment has led to the emergence of microorganisms and plants which are resistant to high levels of Cr in their surroundings and this makes them potential candidates for bioremediation of Cr pollution from the environment (Lovley, 1993). They resist the metal by different mechanisms include biosorption, diminished accumulation, precipitation, chromate efflux and reduction of Cr (VI) to Cr (III) (Cervantes *et*

al., 2001). Reduction of Cr (VI) to the less toxic form Cr (III) has been reported by many bacteria (Cervantes, Silver, 1992). *E. cloacae* HO1 strain and *Bacillus sp.* QC1-2 strain were selected by their ability to resist chromate and reduce Cr (VI) to Cr (III) (Campos, 1995). Lovley and Phillips (1994) found a Cr (VI) reducing ability by *Desulfovibrio vulgaris*.*Pseudomonas ambigua, Pseudomonasfluorescens, Pseudomonasputida, Escherichia.coli* and *Bacillus* sp have been also reported as Cr (VI) reducing bacteria (Suzuki *et al.,* 1992; Bopp, Ehrlich, 1988; Ishibashi *et al.,* 1990; Shen, Wang, 1993). Campos *et al.* (1997) and Dvorak *et al.* (1992) reported precipitation of Cr in anaerobic *Clostridium* and by sulfate-reducing bacteria, respectively. Yeast and filamentous fungi can also be used for Cr bioremediation of water and soils contaminated by Cr (Cervantes *et al*., 2001). Chromate-resistant strains of *Aspergillus* spp. (Paknikar and Bhide 1993) and *Candida* spp. Rami *et al.,* 2000) isolated from Cr-contaminated zones showed Cr (VI) reducing activity.

Table 1-4. Comparison between Chromium (VI) and (III)

Property	Chromium 3	Chromium 6	References
Oxidative stress	not highly oxidizing	highly oxidizing	(Shanker *et al.*, 2005; Cervantes *et al.*, 2001)
Solubility	less soluble	more Soluble	(Cervantes *et al.,* 2001)
Toxicity	less toxic	highly toxic	

1.1.5. Properties of transposons

Transposons are pieces of DNA that can insert and amplify themselves in a genome. They are also present in all organisms, prokaryotic and eukaryotic; they were first discovered in maize (McClintock, 1948). Also, they make up a high proportion of genome volume and move from one genomic position to another by a cut-and-paste mechanism (Muñoz-López, García-Pérez, 2010).

They have multiple modes of movement or transposition (Fig 1.4, Settles, 2009). They can also move between the chromosome and the various plasmids and temperate phage. DNA can be transferred and transposed bacterial cell as well as between unrelated bacteria in a population; thus, transposons have had an important role to play in bacterial evolution (Berg, Sasakawa, 1984)

Figure 1. 4. Methods of transposon movement
(Http://www.broadinstitute.org/files/news/stories/full/transposons_720x720_v2.jp

When a transposon ‘jumps’ into a gene, it may cause disturbance to that gene and possibly changes the phenotype of the organism. Transposons are valuable tools applied for the genetic analysis of bacteria (Engels, Berg & Howe, 1989). If the transposon inserted between genes (Fig 1.5A), the structure of the proteins produced by the surrounding genes will not affected. If the transposon inserted within the gene, the gene expected to lose its

function Fig 1.5 (B). If the transposon inserted between the promoter and the start of the gene (Fig 1.5 C), the expression of the gene will be affected.

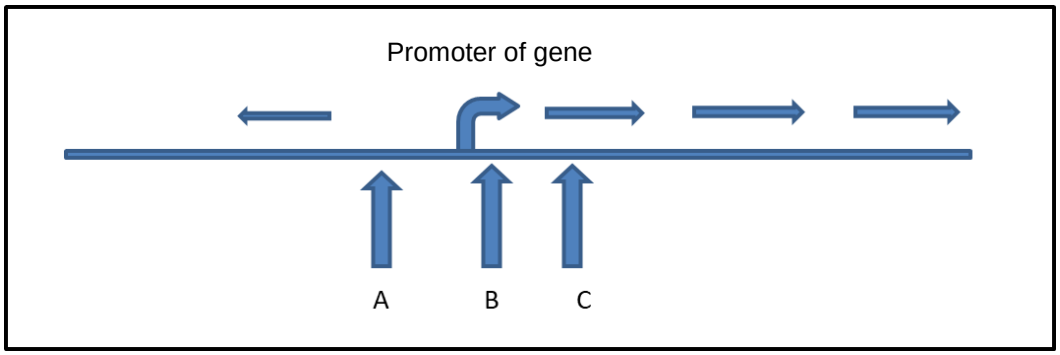


Figure 1.5. Different locations that the transposon may insert around protein-encoding genes.

Transposable elements may also make up a substantial proportion of a cell's genome (Stein *et al*., 2003). Transposons are valuable tools applied for the genetic analysis of bacteria (Engels, Berg & Howe, 1989). Transposable elements may also make up a substantial proportion of a cell's genome (Stein *et al*., 2003).

According to the nature of the intermediate that is used for mobilization, the mobilization of TEs is called transposition or retro-transposition. It has been suggested that there are many methods in which the activity of TEs can positively and/or negatively impact a genome; for example, TE mobilization can support gene inactivation, modify gene expression or prompt illegitimate recombination. Therefore, TEs have played an important role in genome development (Muñoz-López, García-Pérez, 2010)

DNA transposons, for instance, can change or inactivate the expression of genes when inserted within the coding sequences of genes or in regulatory regions (Clegg, Durbin, 2003). Also, by the mobilization of non-transposon DNA, TEs can contribute to the reorganization of a genome. Likewise, TEs can participate in the loss of genomic DNA by internal deletions (Petrov, Hartl, 1997).

There are two types of TE: RNA transposons (Class I) and DNA transposons (Class II). A type of TE, RNA transposons (Class I), functions by the reverse transcription of an RNA intermediate depending on the presence of Long Terminal Repeats (LTR) that are divided into two main groups (Fig 1.6). DNA transposons (Class II) are usually transferred by a cut-and-paste mechanism in which the transposon is removed from one location and reintegrated elsewhere. Most DNA transposons move through a non-replicative mechanism. DNA transposons contain a transposase gene that is flanked by two Terminal Inverted Repeats (TIRs) (Fig 1.6) (Muñoz-López, García-Pérez, 2010).

Figure 1-6. Classes of transposable elements (TEs)

A Class I element (clade LINE-1) consists of a 5'-UTR with an internal promoter activity, and two Open Reading Frames (ORFs). ORF1 encodes a nucleic acid binding protein, while ORF2 encodes a protein with Endonuclease (EN) and a Reverse Transcriptase (RT) activity which lacks Long Terminal Repeats (LTR), and ends in a poly (A) tail (Class II elements which consist of a transposase gene flanked by Terminal Inverted Repeats (Muñoz-López, García-Pérez, 2010).

DNA transposons are classified into two different families depending on their sequence: TIRs, and TSDs. The families in Subclass I are Tc1/*mariner,* PIF*/Harbinger*, hAT, Mutator, Merlin, Transit, P, piggyBac, and CACTA, Helitron and Maverick transposon (Muñoz-López, García-Pérez, 2010). The superfamily Tc1/mariner is probably the most extensively distributed family of TEs in nature; for example, diverse taxa as rotifers, plants, fungi, fish and mammals (Robertson, 1993). The length of Tc1/mariner elements is between 1 and 5 kb, and it encodes a transposase of between 282 to 345 amino acids (Plasterk, Izsvák& Ivics, 1999). Fig 1.7 shown the structure of Tc1/mariner. The mechanism of Tc1/mariner elements is cut-and-paste (Morisato, Kleckner, 1987).

Figure 1-7. The structure of Tc1/mariner transposase

Schematic representation of the Tc1/mariner transposase, which contains a DNA-binding domain with the Helix-Turn-Helix motif (HTH), a Nuclear Localization Signal (NLS) and a catalytic domain with the DDE or DDD motif (Muñoz-López, García-Pérez, 2010).

1.1.5.1. Developing a transposon mutagenesis system for methanotrophic bacteria

Recently, mariner transposon vector pSAM_RI has been used to construct mutants in the *Rhizobiaceae*, *Rhizobiumle guminosarum* bv. viciae 3841 *Sinorhizo biummeliloti*, and *Agrobacterium tumefaciens*, at a high frequency. The percentage of mutant mariner insertion sites in the genome ranged between 68% and 88%. The mean read count per mariner insertion site is detected to have a transposon insert; it started from 18.57 to 22.09 sequence reads per insertion across all replicons with the median read count per

insertion ranging from 10.0 to 13.0 reads per insertion (Perry, Yost, 2014). The pSAM_Rl plasmid (Fig1. 8) was therefore used as the source of the mariner transposon in this work.

Figure 1-8. pSAM_Rl plasmid map.

Unique restriction sites are indicated. Antibiotic markers (ampicillin, AmpR; neomycin/kanamycin, *nptII*), the origin of replication (R6K y oriR), origin of transfer (RP4-oriT), transposase (himar1C9), tranposase promoter. (*rpoD* 5'UTR), *Mme*I-adapted *mariner* inverse repeats (IR_R, IR_L), transposon borne Rho-independent terminator (rrnB T1, rrnB T2). A plasmid map was produced using Snapgene software (Perry, Yost, 2014)

Generally, two methods are applied for identifying the sites of transposon insertion. The first depends on sequencing the around transposon insertion sites (Bishop, Rachwal & Vaid, 2014). The second method relies on complete or almost complete genome sequencing to identify where the transposon has

inserted (Perry, Yost 2014). The two methods have been used for a wide range of bacteria species (Wang *et al.*, 2014).

1.1.5.2. Gene transfer to methanotrophs

Murrell (1994) pointed to a key problem in genetic systems development of methanotrophs. It was the lack of an appropriate transformation method and a means for maintaining heterologous DNA stably in the methanotrophs. Broad host-range plasmids that can function in a wide variety of Gram-negative bacteria, including methanotrophs, can be used to resolve this problem. Numerous large plasmids are identified in methanotrophs; they range from 55 to 190 kb in *Mm, Mcy* and *Ms*. (Lidstrom, Wopat, 1984). In practice, however, the plasmids that have been used in methanotrophs have been derived from other bacteria.

When applied as a transformation method, little success was realized with electroporation of *Mc*. *capsulaus* (Bath) as reported by (Davidson, 1992) and *Ms. trichosporium* OB3b as observed by (Martin, 1994). It was suggested that the power of the electrical impulse was insufficient to permeabilise the cells to DNA. According to Martin, (1994), the presence of extracellular protein or polysaccharide decreases the efficiency of the electric pulse. The most convenient method for transforming methanotrophs with plasmid DNA is the one utilizing conjugation; it can be applied for *Ms. trichosporium* OB3b (Martin, Murrell, 1995) and *Mm*. *album* BG8, *Methylocystis* POC and *Methylosinus* 6 (Lidstrom, Wopat, 1984).

The most convenient method uses a bi-parental mating technique making use of one *E. coli* donor strains. An in-depth study of gene transfer in *Ms. trichosporium* OB3b was previously carried out by Martin, (1994) utilizing tri-parental mating. The result as reported by Keen *et al*. (1988) was the isolation of transconjugant methanotrophs at an isolation frequency of 10^{-8} to 10^{-9} for the extensive host range of plasmids including pDSK509 and pVK I00 as observed by Knauf and Nester (1982).

As described by Simon, Priefer & Pühler (1983) the technique utilizing conjugation can be modified employing *E. coli S17-1* that carries the RP4

transfer functions which are integrated into the genome, to allow conjugation via biparental mating. The result is a highly effective conjugal transference of plasmids which carry the RP4 *mob* sequence. Martin (1994) found that the optimization of the filter mating conditions resulted in the constant transformation frequencies of 10^{-7} to 10^{-8} for pDSK509 and pVKI00. Martin and Murrell (1995) observed that such conversion technique is used with a great success to transmit plasmids from *E. coli* S17-1 to *Mm. album* BG8, *Mcy. parvus* OBBP and *Ms. trichosporium* OB3b.

Lloyd *et al*. (1999) reported by using a homologous expression system that the expression of sMMO was restored in sMMO-minus mutants by complementation with plasmid-encoded genes. This system has the advantage of a high level of active expression, arising from the natural sMMO promoter (s). Also, homologous expression system more recently a similar study has been used to investigate the roles of specific amino acids in the hydroxylase component of sMMO in substrate oxidation and control of regioselectivity (Smith, Nicoll, 2018). Perry, Yost (2014) transformed the plasmid pSAM_R1 into E. *coli SM10λ* pir via electroporation, and then transformed it from E*. coli SM10λ* pir to Rhizobiaceae via conjugation. They identified novel genes associated with alginate production in *Pseudomonas aeruginosa* using mariner transposon-mediated mutagenesis.

1.2. Aims of the work reported in this study

The overall aim of this study was to develop a transposon mutagenesis system for methanotrophic bacteria and use it to identify genes that are responsible for remediation of chromium (VI) and selenium $SeO3^{2-}$.

The main objectives were as follows:

1- To develop a system for transposon mutagenesis of methanotrophs by using the mariner transposon in pSAM_R1.

2- To create a library of mutants of Ms. trichosporium OB3b.

3- To use transposon mutagenesis to identify genes that are responsible for the remediation of selenite SeO32- in the methanotrophs.

4- To investigate the physiology of selenite reduction.

Chapter 2 Method development for monitoring chromium and selenite bioremediation by methanotrophs

2.1. Introduction

Research has shown that the methanotrophs can reduce some heavy metals such as chromium (VI) and selenite (SeO_3^{2-}) from highly toxic to less toxic forms (Al Hasin, *et al*., 2009; Eswayah, *et al*., 2017). Chromium (VI) is one of the heavy metals which is highly toxic, that may be present in the all phases of the environment such as air, soil and water (Tugarova, *et al*., 2014). There is one strain of methane oxidising bacteria for which there is a published report of reduction of chromium (VI) to chromium (III). It was found that the strain *Mc. capsulatus* (Bath) was able to reduce chromate (VI) from 1.4 mg L^{-1} to 1000 mg L^{-1} because of its ability to remediate chromate by adding potassium chromate (VI) to the test media, while the genus *Ms. trichosporium* OB3b could not reduce this metal at the same concentration (Al Hasin, *et al.,* 2009).

Selenium is also a toxic element in the environment and exists in different natural oxidation states. Most organisms need trace amounts of selenium for growth and metabolism. Recent research discovered that *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b are both able to reduce the toxic selenite (SeO_3^{2-}) to red elemental selenium (Se^O) (Eswayah*, et al.,* 2016). One of the purposes of this study was to identify which gene(s) are responsible for reduction of selenite to elemental selenium.

In a previous study, *Streptomyces sp* was investigated under aerobic conditions. The result showed that the *Streptomyces sp* are able to reduce Se (IV) (selenite) and Se (VI) (selenate) to less toxic Se (0) with the formation of Se nanoparticles (SeNPs) (Tugarova *et al*., 2014).

In general, the optimum pH values for growth of methanotrophs in soils and methane oxidation are consistent with the optimal pH values for the growth of most known methanotrophs in pure cultures (Dunfield, Dumont & Moore, 1993). The methane oxidation was observed in soil samples from pH 3.5 to 8.0, and in peat samples between pH values of 3.7 to 4.4, but the pH optimum for methane oxidation in acidic peats was pH 6.0 to 7.0. The methane-oxidizing microflora in these acidic environments were neutrophilic methanotrophs. In some acidic peat samples, the rates of methane oxidation were only slightly

influenced by pH at values between 4.0 and 6.0 and reduced at values below and above this range, suggesting that methanotrophic populations are adapted to acidic environments (Hanson, Hanson 1996). More recently, verrucomicrobial methanotrophs have been isolated that are able to grow at pH < 2 (Pol *et al.,* 2007; Dunfield *et al*., 2007).

This study aims to identify specific genes that are responsible to reduce toxic chromium (VI) and selenite (SeO_3^{2-}). To start this project, the first step was to confirm that the methane oxidising bacteria are able to reduce toxic chromium (VI) and selenite and then to evaluate the tests used to identify each of these activities in order to decide which element would be the easiest to establish a workable high throughput screen for mutants that were not active in the detoxification of the element.

2.2. Materials and methods

2.2.1. Bacterial Strains and Growth Conditions

Two strains of methanotrophic bacteria *Ms. trichosporium* OB3b and *Mc. capsulatus* (Bath) were obtained from Microbiology laboratory of Sheffield Hallam University. The strains were grown and propagated aerobically in sterile nitrate mineral salts (NMS) media (Smith & Murrell 2011) using methane (1:4 v/v in air) as the source of carbon and energy. The NMS media contained (per L of deionized water) KNO_3, 1000 mg; $MgSO_4.7H_2O$, 1000 mg; $CaCl_2.2H_2O$, 200 mg; $NaMoO_4.2H_2O$, 0.5 mg; Fe-EDTA, 3.8 mg; $CuSO_4.5H_2O$ 0.1 mg; $FeSO_4.7H_2O$, 0.5 mg; $ZnSO_4.7.H_2O$, 0.4 mg; H_3BO_3, 0.15 mg; CoCl3.6H2O, 0.05 mg; Na2EDTA, 0.25 mg; MnCl2.4H2O, 0.02 mg; $NiCl_2.6H_2O$, 0.01 mg; Na_2HPO_4, 497 mg; KH_2PO_4, 390 mg. The experiments were performed in 50 mL liquid cultures in 250mL conical Quickfit® flasks capped with Suba-Seals (Sigma-Aldrich) to prevent methane loss while allowing the addition and removal of material using hypodermic syringes. The *Ms. trichosporium* OB3b and *Mc. capsulatus* (Bath) cultures were incubated at the optimum growth temperature of 30 and 45°C, respectively, on a shaker at 180 rpm and allowed to grow to an OD_{600} of between 0.5-0.8. Under the conditions used in these experiments, methane was replaced every 3- 5 days for two weeks.

2.2.2. Confirmation of remediation of chromium (VI)

2.2.2.1. Standard curve preparation

Six flasks were labelled and then quantities of potassium chromate solutions (containing 10 mg/L Cr (VI) and 0.18 M sulphuric acid) were added; the components of each flask were mixed (Table 2.1). 0.5 ml of the diphenylcarbazide solution (2.5 mg/l final concertation) was added to each flask and the content was mixed. Flasks were left standing for five min to observe the colour development. Finally, the absorbance of each sample was

measured at $A_{540\ nm}$. The absorbance readings were plotted against the concentration to construct the standard curve.

Table 2-1. Quantities of Cr (VI) and sulphuric acid used for the standard curve

Flask	**Blank**	**1**	**2**	**3**	**4**	**5**
Cr (VI) Solution, ml	0.0	0.4	1.0	2.0	4.0	10.0
0.18 M sulphuric, ml	10.0	9.6	9.0	8.0	6.0	0.0

2.2.2.2. Chromate reduction experiments

The *Mc. capsulatus* (Bath) was cultured to an optical density (OD) of 0.3- 0.8 at $A_{600\ nm}$. 37.3 mg of potassium chromate solution was added to the final concentration of 10 mg/L with respect to Cr (VI) to 50 ml of the culture. Immediately, the first sample was taken and the cells and other particulate materials were removed from the liquid samples by centrifugation (6000 g; 5 min; room temperature). The supernatant was acidified by the addition of three drops of 3 M H_2SO_4, and then 125 µl diphenylcarbazide was added. Next, the tubes were left for five minutes and then the chromium (VI) was quantified by measuring the absorbance in a spectrophotometer at 540 nm. Further samples were taken after zero h, 3 h, 6 h, and 12 h. The negative control that was set up and analysed with same way. NMS media with potassium chromate solution but without bacteria.

2.2.3. Selenite reduction experiment

Ms. trichosporium OB3b and *Mc. capsulatus* (Bath) were grown in ml Quick fit flasks with 50 ml NMS liquid media and incubated with shaking at the 30°C and 45°C, respectively, in the presence of methane as a source of carbon and energy. When the OD_{600} reached 0.7, then 10 µg/ml Se as selenite was added for each strain, accept one flack as a control. The flasks were incubated under the same conditions for 72 h. Both methanotrophs were also grown on NMS

agar supplemented with 10 µg/ml Se as selenite for one week at 30°C and 45°C respectively in a static incubator.

To determine whether the pH of the media of *Ms. trichosporium* OB3b changes with sodium selenite, four samples of *Ms. trichosporium* OB3b were grown in flasks with 50 ml of NMS media, then the flasks were incubated with shaking at 30°C in presence of methane. A 5 ml sample from each culture was taken when the OD_{600} reached ~ 0.7 and immediately 10 µg/ml Se as selenite was added each sample. The samples were centrifuged, and then the supernatant from each sample was used to measure the pH. The pH was measured again when the colour of media changed to orange.

2.3. Results

2.3.1. Confirmation of chromium (VI) reducing activity in the methanotrophic bacterium; *Mc. capsulatus* (Bath)

2.3.1.2. Standard curve of chromium (VI) concentration

Quantities of potassium chromate solutions for each sample were mixed and the diphenylcarbazide solution was added as indicator. The colour that was observed depends on the concentrations of chromium in the solutions. Absorbance was measured for the samples at 450 nm at different concentrations of chromium, and then the readings were recorded as displayed in (Table 2.2) and then applied as in (Fig 2.1). This was used to calculate the concentration of chromium (VI) reduced by *Mc. capsulatus* (Bath) in the experiment

Table 2-2. Chromium concentration and Optical density (OD)

Chromium concentration mg/l	**Absorbance at 450 nm**
0	0
0.4	0.038
1.0	0.106
2.0	0.219
4.0	0.449
10.0	1.69

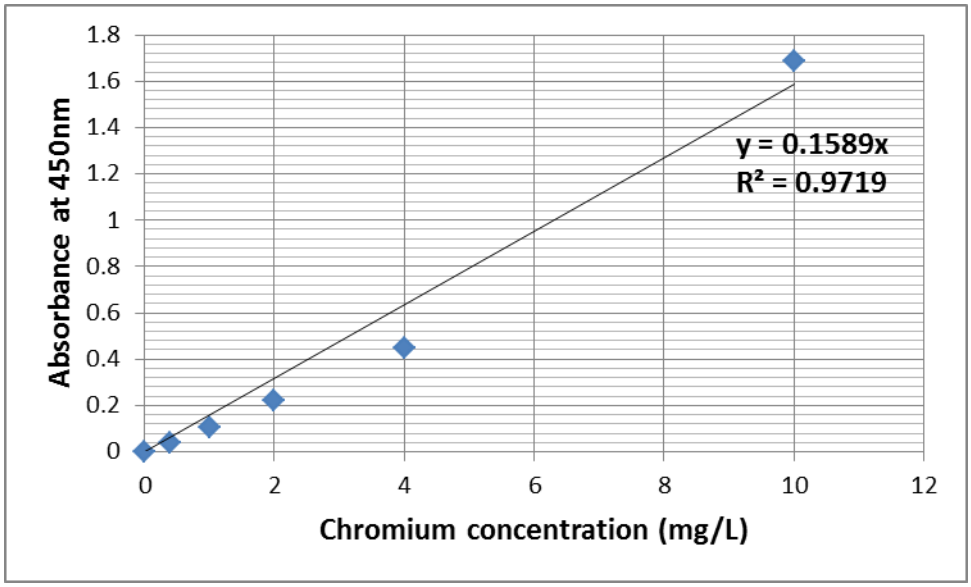


Figure 2-1. Standard curve of Cr (VI) measurement using the diphenylcarbazide assay

2.3.2. The ability of *Mc. capsulatus* (Bath) to reduce chromium (VI)

The ability of *Mc. capsulatus* (Bath) to reduce chromium VI was tested by adding potassium chromate (VI) to the culture. As illustrated in Figure 2.2 the red curve exhibits the concentration of chromium in the culture of bacteria. One ml of the supernatant was collected every 3 h for 12 h. The concentration started from 10 mg/l at zero time and at the end of the experiment, it reached 2.5 mg/l as in Table 2.3 after comparison with standard curve of Cr in the above, and then applied as a curve as in Fig 2.2. This means that *Mc. capsulatus* (Bath) could reduce chromium (VI) while the blue curve reveals the concentration of chromium in the medium without the bacteria (control). There were no changes in the chromium concentration in the control during the 12 hours period. This confirms that decline in chromium (VI) concentration in the experimental flask was due to the *Mc. capsulatus* (Bath).

Table 2-3. Chromium concentration and Optical density (OD)

Time h	Culture without bacteria (Control)		Culture with bacteria	
	Absorption of sample at 450 nm An	Concentration of chromium compared with standard curve of Cr (VI) mg/l	Absorption of sample at 450 nm	Concentration of chromium compared with standard curve of Cr (VI) mg/l
0	1.6	10	1.6	10
3	1.6	10	1.4	8.5
6	1.6	10	0.9	5.5
9	1.6	10	0.8	5
12	1.6	10	0.4	2.5

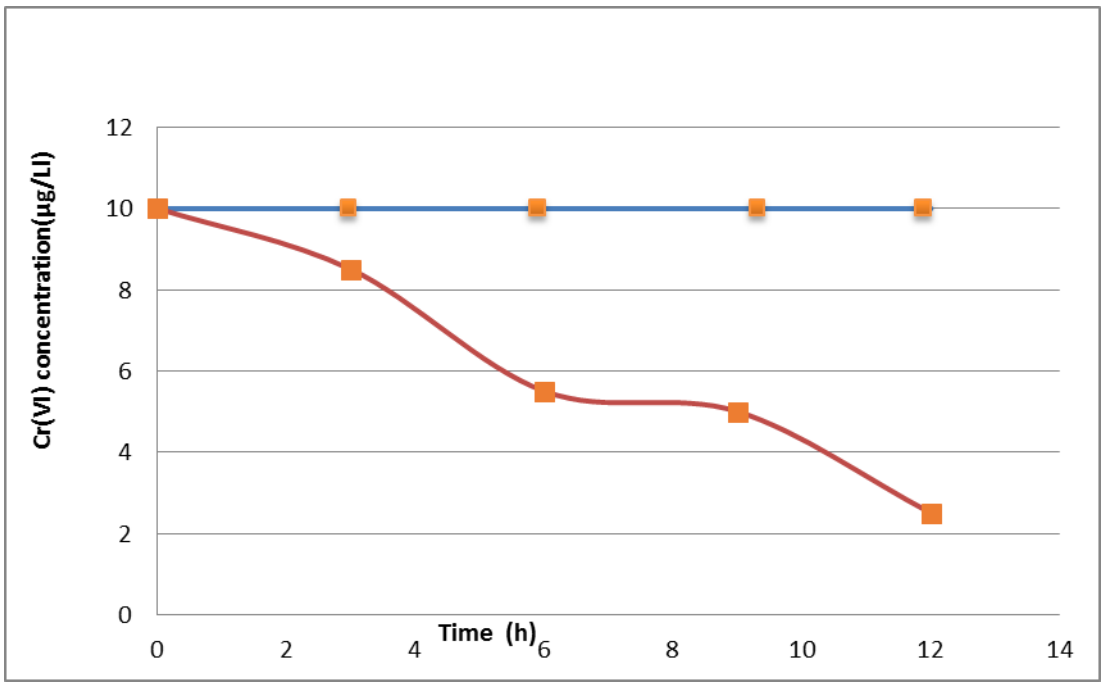


Figure 2-2 Reduction of chromium (VI) by *Mc*. *capsulatus* (Bath).

2.3.3. Capacity of *Ms. trichosporium* (OB3b) to reduce selenite (SeO_3^{2-})

The ability of *Ms. trichosporium* OB3b to reduce selenite was tested by adding 10 µg/mL Se as Na_2SeO_3 into the NMS liquid and solid media in the presence of methane.

After three days of incubation, the colour of the liquid media changed to orange (Fig 2.3 B) and this was also observed solid NMS media where bacterial colonies changed colour to orange (Fig 2.3 D). In contrast, the control did not show any phenotype changes (Fig 2.3 A, and C). Hence it was confirmed that *Ms. trichosporium* OB3b can reduce SeO_3^{2-} to red coloured Se^0

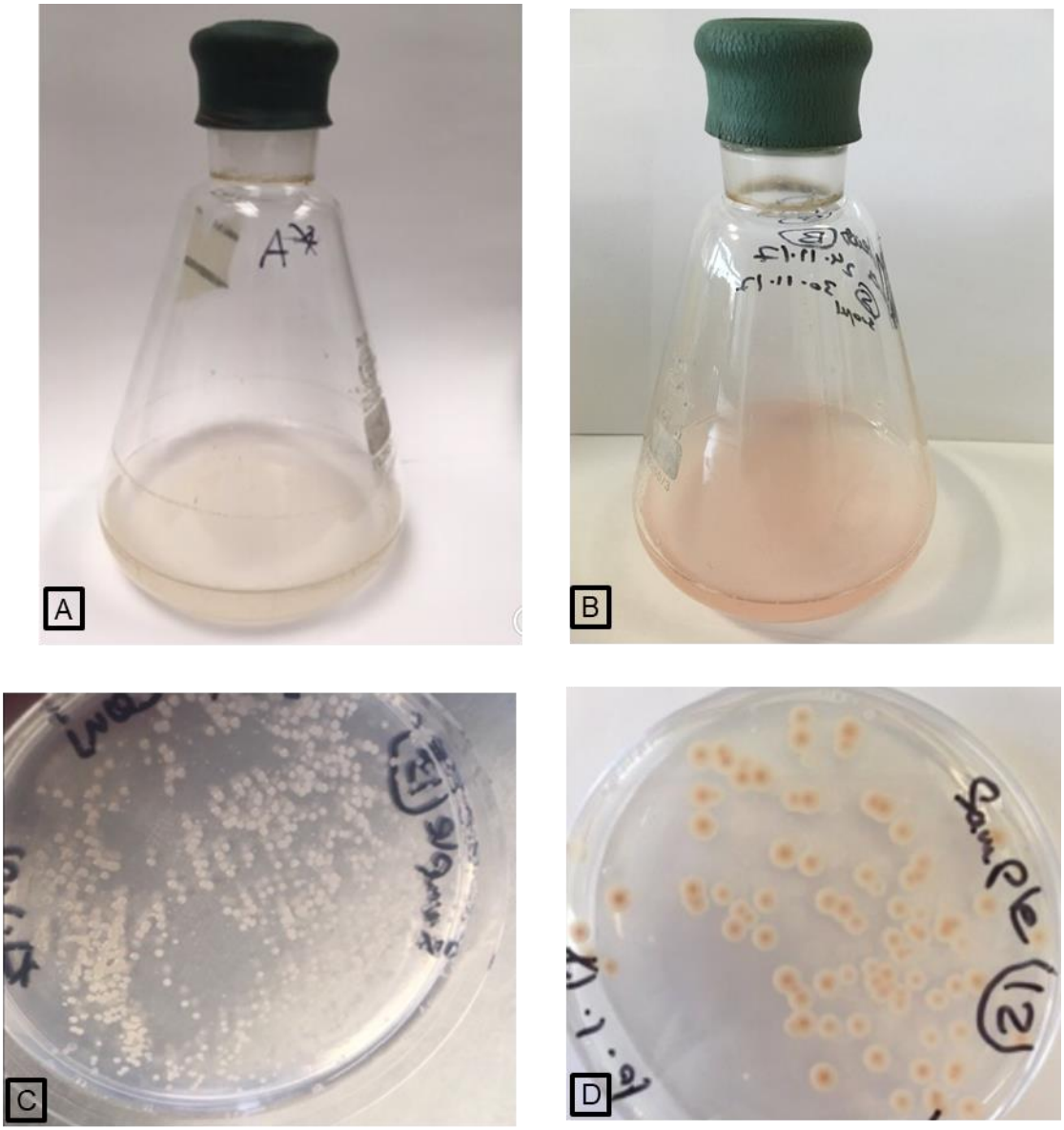


Figure 2-3. Reduction of selenite by *Ms. trichosporium* OB3b in liquid media.

Ms. trichosporium OB3b without selenite as a control. (A*) Ms. trichosporium* OB3b with selenite (B), and solid media, *Ms. trichosporium* OB3b without selenite as a control (C), *Ms. trichosporium* OB3b with selenite (D)

2.3.4. pH from the supernatant of the culture of *Ms. trichosporium* (OB3b) before and after adding the selenite

The pH value in the NMS media did not show a substantial change with/without sodium selenite (Table 2.4). The pH values decreased in the samples 1 and 2 after adding sodium selenite, while the pH of the other samples increased in 3 and 4 (Fig 2.4). As expected, the pH did not change much due in presence of the buffer phosphate which is included the NMS media and will minimise pH changes in the culture.

Table 2-4. pH of the supernatant of the culture of *Ms. trichosporium* (OB3b) before and after adding selenite.

Samples	**pH before selenite adding**	**pH after selenite reduction**
1	7.14	7.06
2	6.90	6.82
3	6.87	6.81
4	7.04	7.10

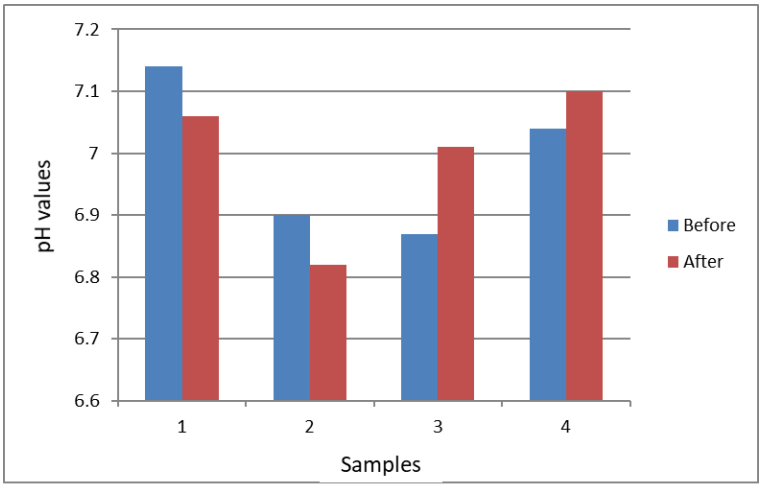


Figure 2-4. pH before and after adding the selenite in the media of *Ms. trichosporium* (Blue; before), (Red; after).

2.4. Discussion

The results in this chapter are consistent with two previous studies which have reported that methanotrophs are able to bioremediate the highly soluble toxic chromium (VI) to the less toxic chromium (III) (Al Hasin *et al*. 2009), and also are able to reduce the toxic selenite (SeO_3^{2-}) (but not selenate [SeO_4^{2-}]) to red spherical nanoparticulate elemental selenium (Se0) (Eswayah *et al*. 2017).

The present results confirm that 10 mg/L starting concentration of potassium chromate was reduced to 2 mg/L by *Mc. capsulatus* (Bath) after 72 hours of incubation in the presence of methane as a sole carbon source (Fig 2.2). This is consistent with the observation of Al Hasin *et al* (2009) that *Mc. capsulatus* (Bath) can reduce chromium (VI) to form chromium (III) at concentrations of chromium from 1.4 to 1000 mg/l. Chromium (VI) reduction by whole cells of *Ms. trichosporium* OB3b was not investigated here because the previous study Al Hasin *et al* (2009) did not find reduction of chromium (VI) by this strain over 200 hours period of incubation.

Studies conducted in this chapter confirmed the ability of *Ms. trichosporium* OB3b strain to reduce the soluble toxicity of selenite. The results showed that the growth of *Ms. trichosporium* OB3b with 0.5 mg/L selenite in the liquid and solid media is accompanied by a change to a red colour of the colonies on the plates and liquid cultures (Fig 2.3 B& D). This result is consistent with a previous study (Eswayah *et al.,* 2017), where it was found that the methanotrophs *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b can bioremediate the toxic of selenite (SeO_3^{2-}) but not selenate (SeO_4^{2-}) to red spherical nanoparticulate elemental selenium (Se^0). Also, it was reported that the minimum inhibitory concentration (MICs) for selenite was70 mg/l for *Mc. capsulatus* (Bath) and 5 mg/l for *Ms. trichosporium* OB3b. However, the bacteria grew well with increase in the selenite concentration and the phenotype of bacteria was not changed although the colour of colonies was red. Other microorganisms have been reported to reduce selenite, including *Azospirillum brasilense* , (Tugarova *et al.* 2014).

The tested pH values of the media used to culture *Ms. trichosporium* OB3b before and after adding the selenite did not show high variation because of the buffer phosphate that is a component of nitrate mineral salts (NMS) medium, which buffers against any pH changes produced by the bacteria. pH values in samples 1 & 2 (Fig 2.4) decreased after adding the selenite while the other two samples showed a modest increase in pH. This difference may be because the numbers of cells were different for each sample.

To conclude, these results enabled the study to identify the genes which are responsible for the remediation of selenite $(SeO_3)^{2-}$ in the methanotrophic bacteria, and to detect the position of selenite reduction in the cell of *Ms. trichosporium* OB3b. They showed that the production of red elemental selenium from selenite is a reaction that can be easily observed in liquid culture or in colonies on selenite-containing plates, which is suitable for screening many clones on agar plates. In contrast, detection of the chromium (VI)-reducing activity requires a multistep spectrophotometric assay of the chromium (VI) and so was judged less suitable for easy screening of many mutant clones.

Chapter 3 Development of the protocol for transposon mutagenesis and construction of the first transposon library in *Methylosinus trichosporium* OB3b

3.1. Introduction

In order to be able to use the colour based screening method for identifying mutants deficient in selenite reduction, it was necessary to have a system to produce a suitable library of gene-inactivated mutants.

Mobile genetic elements (MGEs) are pieces of DNA that encode proteins that mediate the movement of DNA within genome or between bacterial cells. MGEs are active agents of bacterial evolution for the most part acquired by horizontal gene transfer (Frost *et al*., 2005). Transposons are one type of (MGEs) that can insert and amplify themselves in a genome. They can also move between chromosomes and various plasmids and temperate phage DNAs present in a bacterial cell as well as between unrelated bacteria (Berg, Berg & Sasakawa, 1984). To introduce DNA into the cells in the lab, electroporation can be used, where a high-voltage electric field created by the Bio-Rad Gene Pulser, a capacitor discharge device, is used to permeabilise the cell to DNA (Chang, 1989). Miller, Dower & Tompkins (1988) were developed protocols for efficient introduction of plasmid DNA into E. *coli* via electroporation. Electroporation has also been used successfully to deliver DNA into most human tissues *in vivo* (Heller, 2006).

The technique of insertion sequencing (INSeq) combines the use of a transposon with high-throughput sequencing to allow characterisation of a large number of mutants (typically in the tens of thousands, or more) to study gene function at the genome level (van Opijnen& Camilli 2013). Many studies have used an insertion sequencing (INSeq) technique; for example, Perry and Yost (2014) used the INSeq technique with plasmid pSAM_RI (used in the current work) to explore the whole genome of a bacterium from the *Rhizobiaceae* to identifying new essential rhizosphere colonization genes, also identifying mutations that provide a phenotypic growth advantage. They used conjugation to transfer the plasmid pSAM_R1 from *E. coli SM10λpir* to *Rhizobiaceae,* and their studies succeeded to insert the mariner transposon into the genome of the *Rhizobium leguminosarum* randomly, and the mutagenesis was at a high frequency to generate saturation libraries of transposon insertion mutants.

Bharani *et al*. (2014) also used the INSeq methodology. They used the *E. coli S17* carrying the transposon IS-O-Km/hah as donor and *Pseudomonas* strains *Pph*1302A and 1448Aas a recipient for conjugation. They identified the important genes in the *Pseudomonas syringae* by using *in vitro* screening of transposon libraries.

In this chapter methods were developed to allow the plasmid pSAM_R1, which contains the mariner transposon, to disrupt the chromosomal DNA of *Ms. trichosporium* OB3b. Before this was possible the plasmid needed to be transferred to *E. coli SM10λ pir*, because this strain is able to replicate the plasmid and produce a pilus to pass the plasmid easily to *Ms. trichosporium* OB3b as recipient via the conjugation process. The *E. coli SM10 λ pir* was used because it encodes the pilus required for conjugation and can also replicate the plasmid pSAM_R1 because it possesses the *λ pir* prophage (Perry and Yost, 2014).

3.2. Material and methods

3.2.1. Bacterial strains, plasmids, media and culture conditions

Strains of methanotrophic bacteria, *E. coli SM10λpir* and three types of plasmids, as in illustrated in Table 3.1 were used in this study, figures 3,1 and 3,2 showing the diagram of plasmids pTJS175 and pET23a+.

Table 3-1. Bacterial strains and plasmids

Name of bacterium/plasmid	**Description**	**Reference, Source**
Methylococcus capsulatus (Bath) *Methylosinus trichosporium* OB3b	Gamma proteobacterial. *Alphaproteobacteria*	Microbiology laboratory of Sheffield Hallam University
E. coli SM10λpir	Encodes pilus required for conjugation and has the *λpir* prophage.	Biomedal Company
pTJS175	Contains the whole sMMO operon from Ms. trichosporium in the vector pTJS140; confers resistance to ampicillin	Smith *et al.* 2002
pET23a+	3666 bp plasmid conferring ampicillin resistance	Gift from David Smith, Sheffield Hallam University
pSAM_R1	Contains the Mariner transposon	Perry and Yost 2014

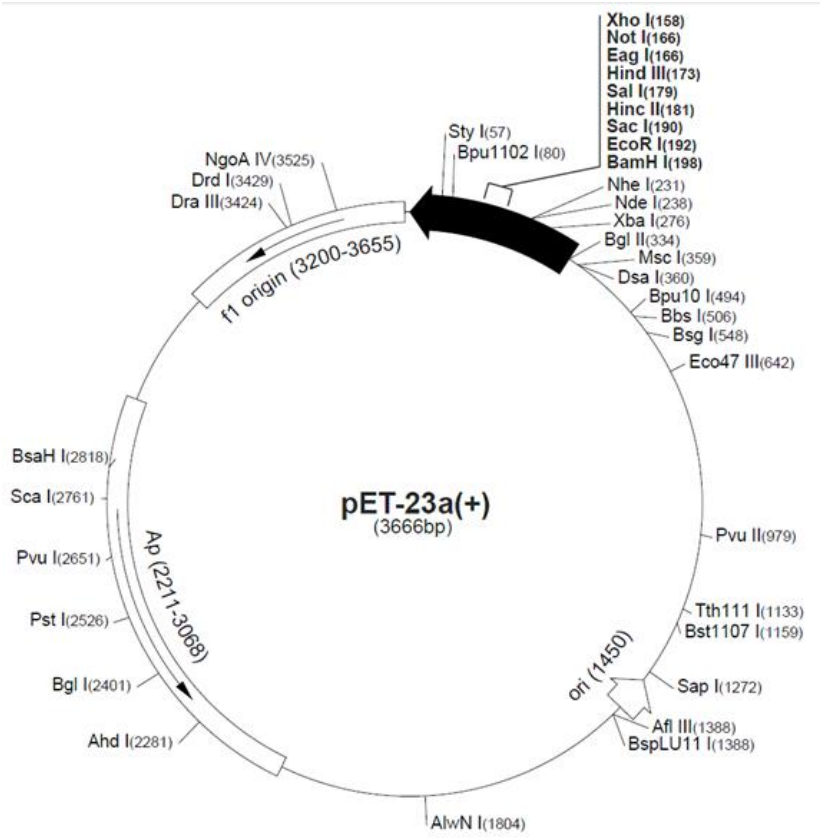


Figure 3-1. Diagram of pET23a+ plasmid

Figure 3-2. Diagram of pTJS175 plasmid

Three types of restriction enzymes and two types of antibiotics were used in this study as shown in Table 3.2.

Table 3-2. Restriction enzymes and antibiotics used in this study

Restriction enzymes	**Recognition sequence**	**Source**
Restriction enzyme *Bam*HI	5' …G^GATCC 3' …CCTAG^G	New England Biolabs
Restriction enzyme *Xho*I	5' …C^TCGAG 3' …GAGCT^C	Promega
Restriction enzyme *Nde*I	5' …C^ATATG 3' …GTATA^C	New England Biolabs
Antibiotics	**Description**	**Source**
Kanamycin (Km)	To select *E. coli SM10λ pir* to grow	Sigma
Ampicillin (Amp)	To select for plasmids in *E. coli*SM10λpir	Sigma

E. coli SM10λpir was cultivated on LB agar plates at 37°C for 16 hours and, where appropriate, ampicillin was used at a final concentration 100 µg/ml. Small-volume culture of *E. coli SM10λpir* and plasmid-containing derivatives for preservation were prepared by inoculating a single colony into 5 ml of LB broth (containing 100 µg/ml of ampicillin where appropriate) and incubating with shaking at 37°C for 16 hours. *E. coli SM10 λpir* and derivatives containing plasmids were grown in small volume culures as described above and stored at -80°C with glycerol. The appropriate volume of glycerol (to give a final concentration of 30 % v/v) was added to a suspension of log-phase bacteria to minimise damage on freezing and the mixture was vortexed to dissociate the cells and ensure even mixing of the bacteria with the glycerol. The glycerol-containing cell suspensions were flash frozen in liquid nitrogen before storing at -80°C

3.2.2. Transposon library construction

3.2.2.1. Preparation of Electrocompenent cells

An overnight liquid culture of *E. coli SM10λpir* (5 ml LB agar with 100µg/ml ampicillin) was used to inoculate two flasks each one containing 500 ml LB medium, they were incubated at 37°C while being shaken at 180 rpm until the OD_{600} of the culture reached 0.6 - 0.8. After that the cultures were transferred to centrifuge tubes and put on ice for 15-30 minutes. The cultures were then centrifuged at 8000 ×*g* for 8 min at 4°C. Next the bacterial pellets were re-suspended in a total of 500 ml of sterile distilled cold water and then centrifuged under the same conditions. The pellets were re-suspended in a total of 20 ml of sterile distilled cold water, 10% glycerol and then they were centrifuged at 13000 ×*g* (10 min; 4°C) in Oak Ridge centrifuge tubes. Finally, the last pellet was re-suspended in 2 ml of 10% glycerol. The cell suspension was divided into 50 µl aliquots in Eppendorf tubes, and then stored at -80°C.

Figure 3-3. BioRad MicroPulser electroporation apparatus used in this work

3.2.2.2. Electroporation of *E. coli SM10λpir*

DNA (in a volume of up to 4 µl) from pTJS175, pET23a^{+}, and pSAM_RI were each transformed into 50 µl of prepared *E. coli SM10λpir*. After mixing, the contents of the tubes were transferred to Bio-Rad Electroporation cuvette. DNA solution, cell suspension and electroporation cuvettes were kept on ice at all times. The cells were electroporated by using a Bio-Rad Micropulser with program E c 1 (1.8 kV/5.1 ms) (Fig 3.3). 1 ml of SOC or LB medium was added to the cuvette of electrotransformed *E. coli SM10λpir* cells. The cell suspension was transferred to an Eppendorf tube and then incubated at 37°C while being shaken at 200 rpm for one hour. Aliquots (100 µl) of the transformed cell suspension were inoculated onto LB agar with 100 µg/ml ampicillin and incubated at 37°C overnight.

3.2.3. Plasmid DNA extraction

Plasmid DNA was extracted from overnight liquid cultures using a plasmid miniprep extraction kit (Qiagen, UK). Cultures were prepared by inoculating a single colony into LB broth (10 ml) containing 100 µg/mL ampicillin and incubated at 37°C while being shaken 0.515 ×g overnight. The culture was centrifuged (8000 ×g 3 min; 25°C). The pellets were resuspended in 250 µl of P1 buffer from the kit and transferred into microcentrifuge tubes. 250 µl of the P2 buffer from the kit was added, then the contents of the tube were mixed by inverting the tubes 4-6 times. 350 µl of N3 buffer from the kit was added, and then the contents of the tube were mixed again by inverting the tubes 4-6 times. The tubes were then centrifuged them at 13000×g for 10 minutes. The supernatant was applied to a Qiagen column by centrifugation twice at 13000×g for one minute. The spin column was washed with 750 µl PE buffer from the kit and then centrifuged at 13000×g for one minute. The column was transferred to a clean Eppendorf tube and the DNA was eluted by adding EB buffer (50 µl), then allowing it to stand for one minute. The column was centrifuged again at 13000×g for one minute.

The eluted DNA was then stored at -20°C. The purified DNA was analysed by means of agarose gel electrophoresis. One volume of Loading Dye was added

To 5 volumes of purified DNA and then the solution was mixed by pipetting it up and down before loading onto a TAE buffer (40 mM Tris-acetate, 10 mM EDTA), 0.9% (w/v) agarose gel containing 0.2 µl/ml ethidium bromide. The gels were run for 45-60 min at 95-110 V and visualized under UV light.

3.2.4. QIAprep spin plasmid maxi prep protocol

Plasmid pSAM_RI was purified from the transformed cells of *E. coli SM10λpir* using QIAGEN plasmid maxi prep kit to produce sufficient DNA for analysis of the plasmid. A single colony was used to inoculate a 5 ml LB containing 100 µg/mL ampicillin and grown overnight at 37°C while being shaken at 0.515 ×g. The resulting culture was then used to inoculate a flask with 500 ml LB broth containing the same concentration of ampicillin (100 µg/mL). The flask was incubated overnight at 37°C with shaking at 0.515 ×g. The bacteria were the pelleted by centrifugation at 6000 ×g for 15 minutes at 4°C. The pelleted cells were re-suspended in 10 ml of buffer P1 and mixed thoroughly. An equal volume of buffer P2 was added and the suspension was inverted 4-6 times and left at room temperature for 5 minutes. 10 ml of pre-chilled buffer P3 was added to the mixture and mixed by inversion to stop the lysis reaction. The suspension was incubated on ice for 20 minutes and then centrifuged at 20.000 ×g for 30 minutes at 4°C to remove precipitated chromosomal DNA, denatured protein and SDS. The clear lysate supernatant was centrifuged again for 15 min as stated above in a new sterilized tube to remove any residual precipitate. A QIAGEN-tip 500 column was equilibrated with 10 ml of equilibration buffer QBT and left to run by gravity. The supernatant was then applied to the equilibrated column and the liquid was left to run through the column under gravity. The column was washed with 2 x 30 ml buffer QC. The DNA was eluted from the column with 5ml of buffer QF and precipitated by adding 10.5 ml of isopropanol at room temperature. The tube was then centrifuged at 20.000×g for 30 minutes at 4°C. The plasmid DNA pellet was washed with 5 ml of 70 % (v/v) ethanol, air dried and resuspended with 1 ml of 10 mM Tris-HCl (pH 8.5). The plasmid DNA was then stored at -20°C. P1, P2, P3, QBT, QB, and GF were supplied in the QIAGEN Maxi preparation Kit.

3.2.5. Digestion of Plasmid pSAM_R1

3.2.5.1. Plasmid pSAM_R1

Plasmid pSAM_R1 has four unique restriction sites which are *Nde*I, *Bam*HI, *Xbal* and *Xho*I as illustrated in Fig. 3.4

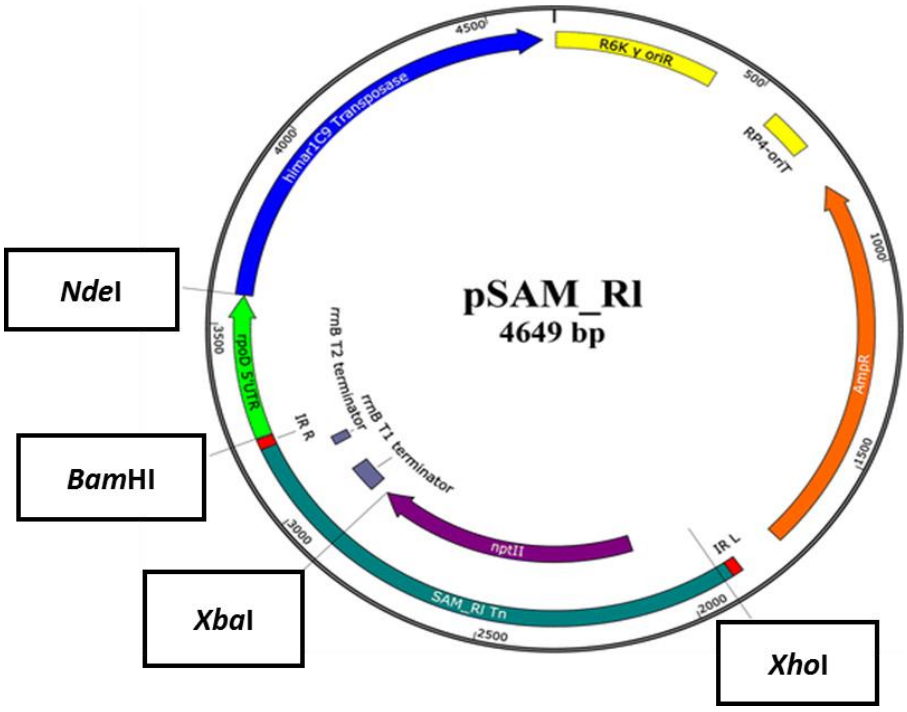


Figure 3-4. Diagram of pSAM_RI plasmid with position of unique restriction enzyme sites.

3.2.5.2. Optimisation of digestion of plasmid pSAM_R1 with *Nde*I + *Xho*I and *Bam*HI + *Xho*I

The plasmid was digested with pairs of restriction enzymes (*Nde*I + *Xho*I and *Bam*HI + *Xho*I). The amount and composition of the digests of plasmid pSAM_R1 is shown in (Table 3.3). Each digest was incubated at 37 ^{o}C for 2 hours, as stated for each experiment. The DNA fragments produced by restriction digestion were separated on 0.9% agarose gels containing 0.2 µl/ml

ethidium bromide and the 1x TAE was used as a gel buffer. The DNA was then visualized under ultra violet (UV) light, and the molecular size markers used were the 1 kb DNA Ladder (Sigma).

Table 3-3. Components of the digest of plasmid pSAM_R1

Component	**Amounts by using *Nde*I + *Xho*I**	**Amounts by using *Bam*HI + *Xho*I**
Plasmid DNA solution	1µl	1µl
Restriction enzyme buffer	1.5µl	1.5µl
Restriction enzyme	for each enzyme 1µl	for each enzyme 1µl
Sterile water	10.5µl	10.5µl
Total	15 µl	15 µl

3.2 6. Conjugation protocol

This technique was used for conjugating plasmids from *E. coli* into the methanotrophs based upon a method developed by Martin (1994). In this study, two strains of methanotrphs, *Mc. capsulatus* (Bath) and *Ms trichosporium* OB3b, were conjugated. Firstly, a 50 ml liquid culture of the methanotroph strain was prepared (OD_{540nm} = 0.5-0.7). An overnight culture of *E. coli SM10λpir* containing the plasmid pSAM_R1 (10 ml) was prepared in LB medium containing 100 µg/ml of ampicillin. The two cultures were mixed and pelleted by centrifugation at 5000 x g for 10 minutes at room temperature. The pellet was washed with 50 ml of a sterile NMS and collected onto a 47 mm sterile nitrocellulose filter (0.2 µm pore size) in a sterile vacuum filter device. The filter was transferred to an NMS agar plates containing 0.02% (w/v) proteose peptone, with the cells uppermost, and the plate was incubated in an

upright position at 37^{o}C for various of incubation 8, 16, 24 hours in the presence of methane (50 % in aerobic conditions).

On the second day, the conjugated cells were collected by washing the surface of the filter with 10 ml of liquid NMS medium and the cell suspension was centrifuged at 5000xg for 10 minutes. The cell pellet was re-suspended in 1 ml of NMS. 100 µl aliquots were plated onto NMS agar with a concentration of kanamycin (concentration stated for each experiment) followed by incubation for 2-3 weeks with methane/air (1:1(v:v)) mixture until single colonies of the methanotrophs appeared.

As detailed below, problems were encountered with the kanamycin selection. Various different concentrations of kanamycin were used (15 µl, 30 µl, 40 µl, 50 µl, 60 µl, 70 µl, 80 µl, 90 µl, 100µl of 15 µg/ml final concentration. In each case, a control of unconjugated methanotroph was used. A number of batches of kanamycin were investigated, and the *E. coli* strain JM109 (which is kanamycin sensitive) grown on LB agar was also used to test the activity of the antibiotic.

Ex-conjugants of *Ms. trichosporium* OB3b were plated on NMS agar containing kanamycin (at 15 and 30 µg/ml final concentration in the presence of methane as the carbon and energy source. The wild type (un-conjugated) strain was used as the negative control.

To analyse the *Ms. trichosporium* OB3b ex-conjugants, the selected isolates were re-streaked on NMS medium containing kanamycin (15 µg/ml final concentration, to select for the plasmid) and nalidixic acid (25 µg/ml final concentration, to eliminate any remaining *E. coli* donor cells). In Ms. trichosporium, the transposon inserted into the chromosome without further manipulation because the replication origin in pSAM_R1 is able to replicate only in strains with the *λ pir* gene (Yost *et al.*, 2014).

3.3. Results

3.3.1. Electroporation of *E. coli SM10λpir*

The electrocompetent cells of the strain *E. coli SM10λ pir* were prepared to transform the plasmids used in this study: pTJS175, pET23a^{+} and pSAM_RI.

3.3.2. Transformation of plasmids pTJS175, pET23a^{+}, pSAM_RI into *E. coli SM10λpir*

The plasmids pTJS175 and pET23a^{+} were tested to confirm the efficiency of the electrocompetent bacteria preparation to take up plasmid DNA before transformation was attempted with the small sample of plasmid pSAM_R1 that was supplied from the research group of Professor Chris Yost. The process of transforming the test plasmids into the electrocompetent *E. coli SM10λpir* cells was successful as judged by growth on the LB agar with 100µg/ml ampicillin, while no colonies were observed on the plates which contained E. *coli SM10λpir* without plasmid (control) (Figs. 3.5 and 3.6). Likewise, the transformation of pSAM_R1 into the same strain was successful (Fig. 3.7). The transformed cells of *E. coli SM10λpir* with plasmid p SAM_RI were stored at – 80°C with glycerol to create the transposon library for *Ms. trichosporium* OB3b.

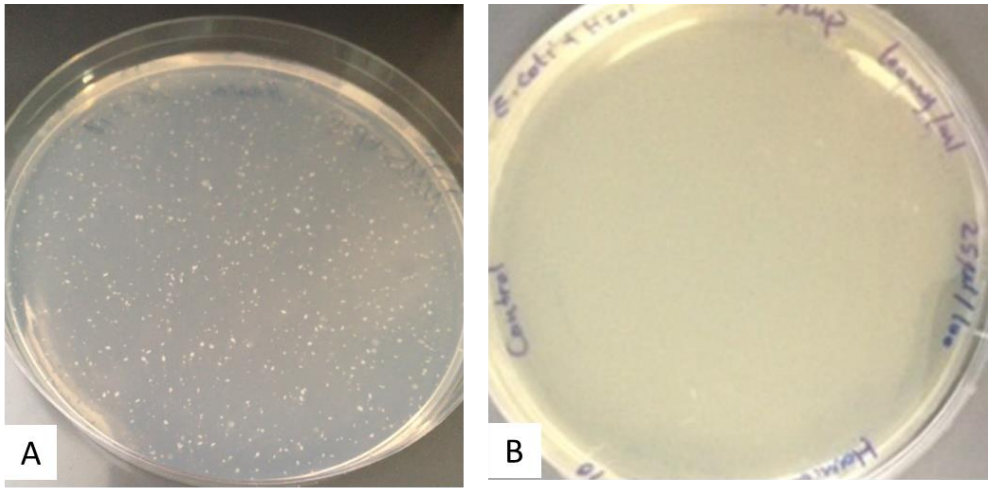


Figure 3-5. Transformed *E.coli* pTJS175 growing on ampicillin agar. (A). No-DNA control transformation plated on ampicillin agar (B).

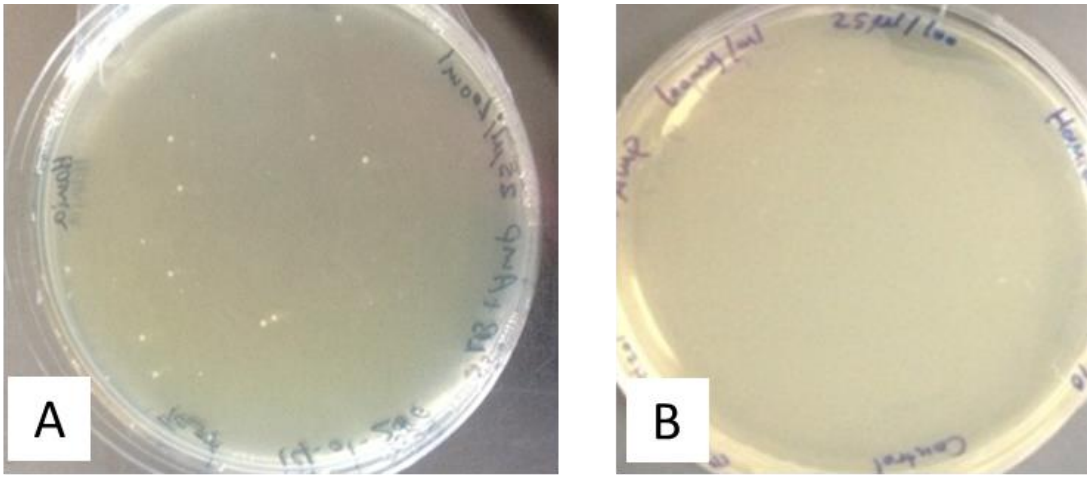


Figure 3-6. Transformed *E.coli* pET23a+ growing on ampicillin agar. (A). No-DNA control transformation plated on ampicillin agar (B).

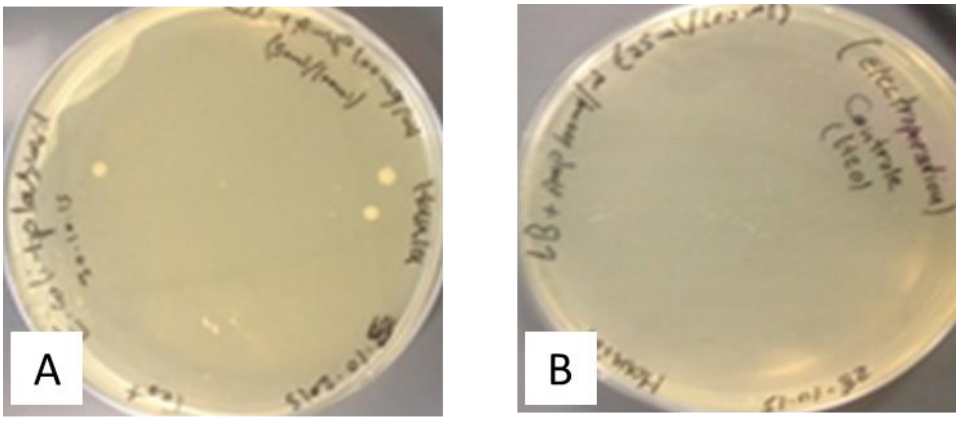


Figure 3-7. Transformed *E. coli* pSAM_R1, transformation growing on ampicillin agar (A). No-DNA control transformation growing on ampicillin agar (B).

3.3.3. Analysis of plasmids isolated from transformed cells

To confirm that the plasmids pTJS175, pET23a^{+} and pSAM_RI had been transformed into *E.coli*, DNA mini-preps were performed using the Qiagen miniprep kit. The purified plasmids were analysed by means of gel electrophoresis. The mobility of the uncut plasmids pTJS175, pET23a^{+} and pSAM_RI were consistent with sizes of about 17,000 bp, 3,666 bp, and 4,649 bp, respectively, and corresponded to the mobility of control preparations of the plasmids where available (Figs. 3.8, 3.9 and 3.10).

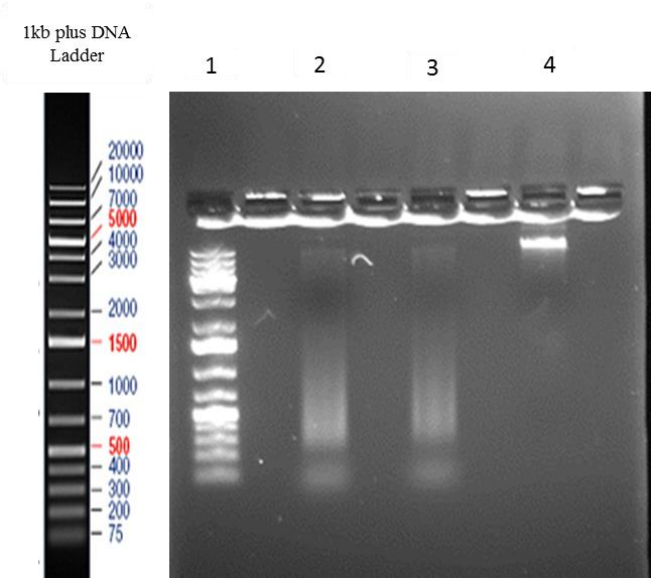


Figure 3-8. Gel electrophoresis of purified plasmid (pTJS175).

Gel electrophoresis of purified plasmid. 1-kb ladder (1), plasmid (pTJS175) preparation from transformed cells (2-3), plasmid control (pTJS175)

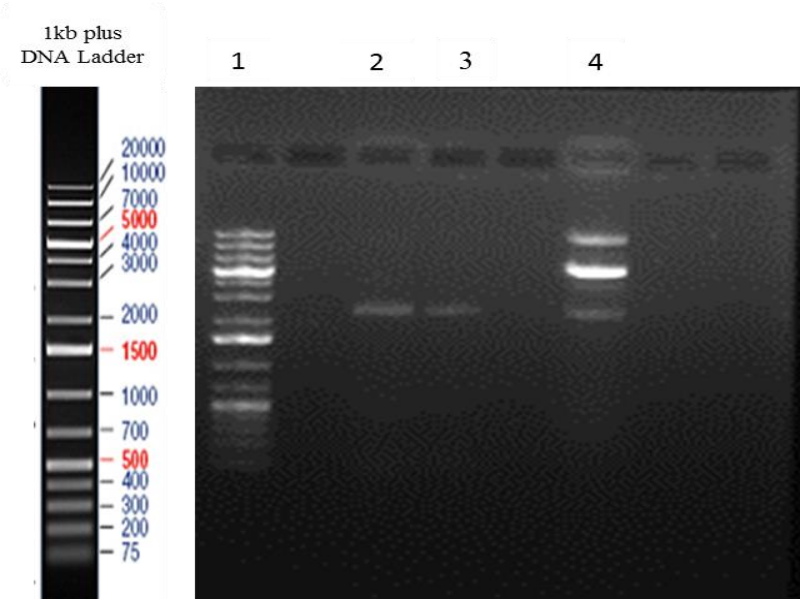


Figure 3-9. Gel electrophoresis of purified plasmid (pET23a+).

Gel electrophoresis of purified plasmid, 1-kb ladder (1), plasmid (pET23a+) preparation from the transformed cells (2, 3), Plasmid control (pET23a+) (4)

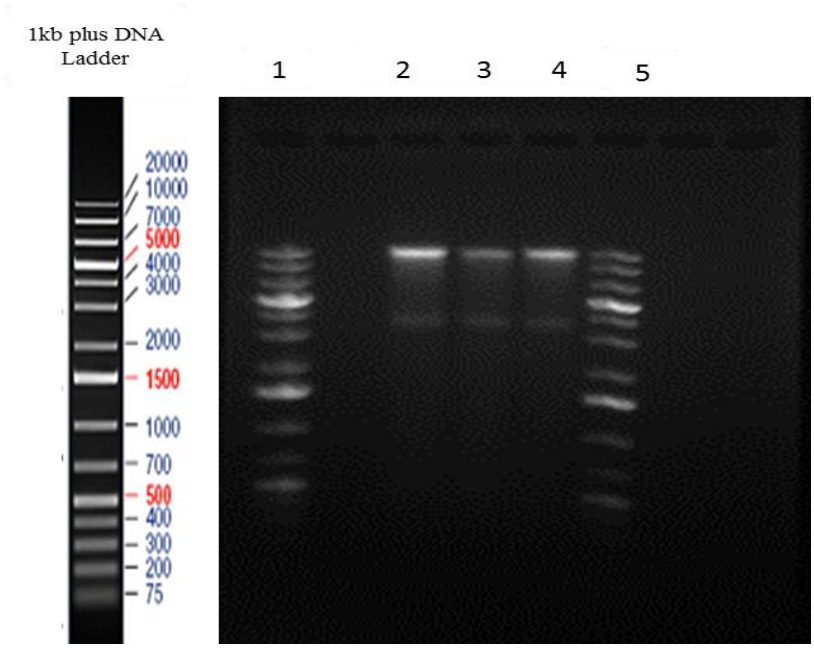


Figure 3-10. Gel electrophoresis of purified plasmid (pSAM_RI).

Gel electrophoresis of purified plasmid, 1-kb ladder (1, 5), plasmid (pSAM_RI) preparation from the transformed cells (2, 3, 4)

3.3.4. Digestion of plasmid pSAM_R1

3.3.4.1 Optimisation of digestion of plasmid pSAM_R1

Restriction digestion was used to investigate whether the transformed cells contained plasmid pSAM_RI, which was needed to generate the transposon library in the present study.

The results from the restriction enzyme digests with the enzymes *Bam*HI, *Nde*I and *Xho*I, as indicated above, were consistent with the previously reported structure of the plasmid (Perry and Yost, 2014). The first experiment Fig 3.12 showed the digestion of plasmid pSAM_R1 with *Nde*I and *Xho*I. The plasmid was digested completely and the molecular weight of the fragments of plasmid pSAM_R1 on the gel were digested as predicted (approximately 3,000 bp and

1,650 bp). The map in Fig 3.11 shows the cutting location of plasmid with the enzymes.

Also, in the second digestion experiment (Fig. 3.14), the molecular weight of the DNA fragments of plasmid pSAM_R1 were as predicted with *Bam*HI and XhoI. Here, the plasmid DNA was digested to give two fragments which were 3.000bp and 1,350 bp. The map of the plasmid in Fig 3.13 shows the location of cutting location of plasmid with the enzymes.

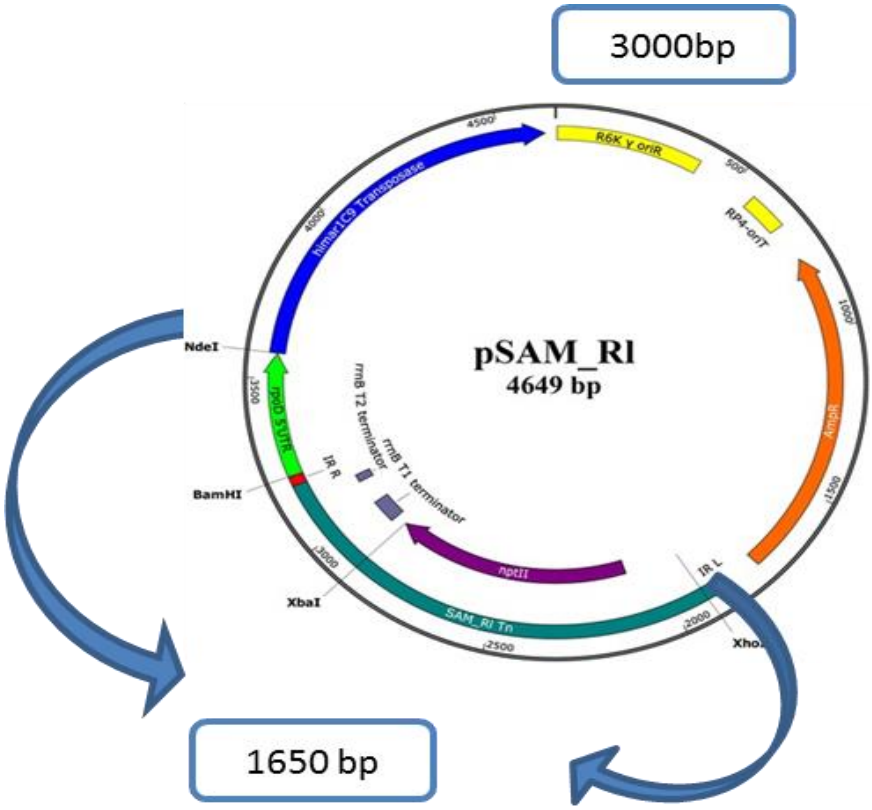


Figure 3-11. Restriction sites of plasmid pSAM_R1 with *Nde*I and *Xho*I

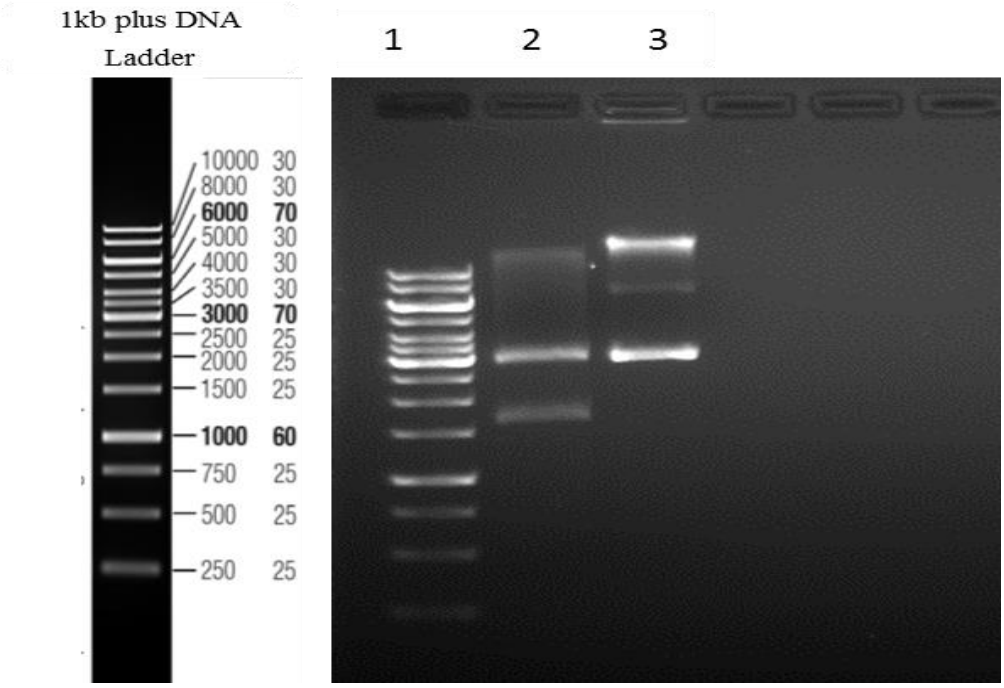


Figure 3-12. The digestion of plasmid pSAM_R1 from the transformed cells, Ladder (1), DNA plasmid prep digested with *Nde*I and *Xho*I (2), DNA plasmid prep without digestion (3)

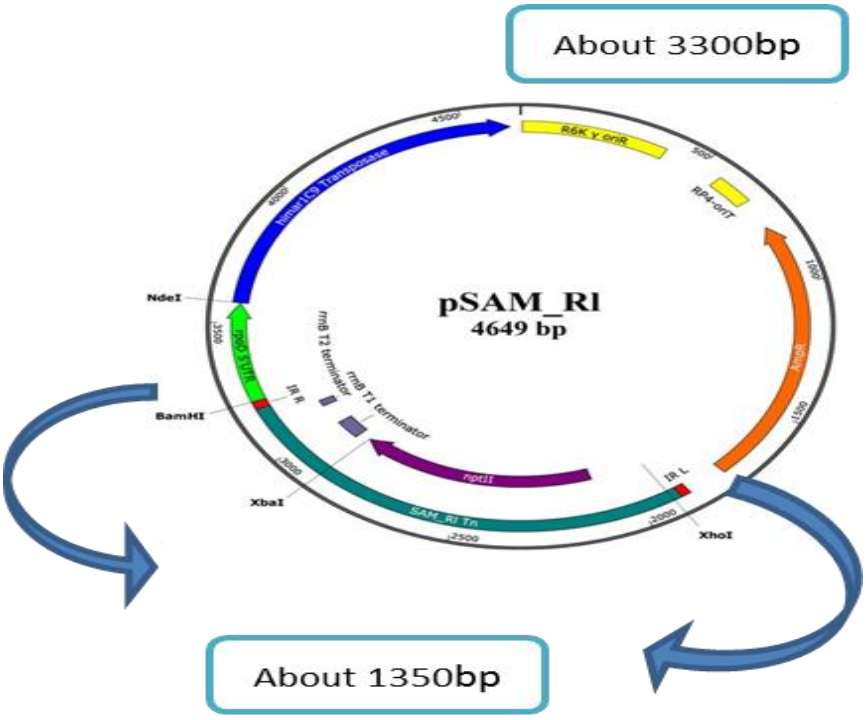


Figure 3-13. Restriction sites of plasmid pSAM _R1 with *Bam*HI and *Xho*I

.

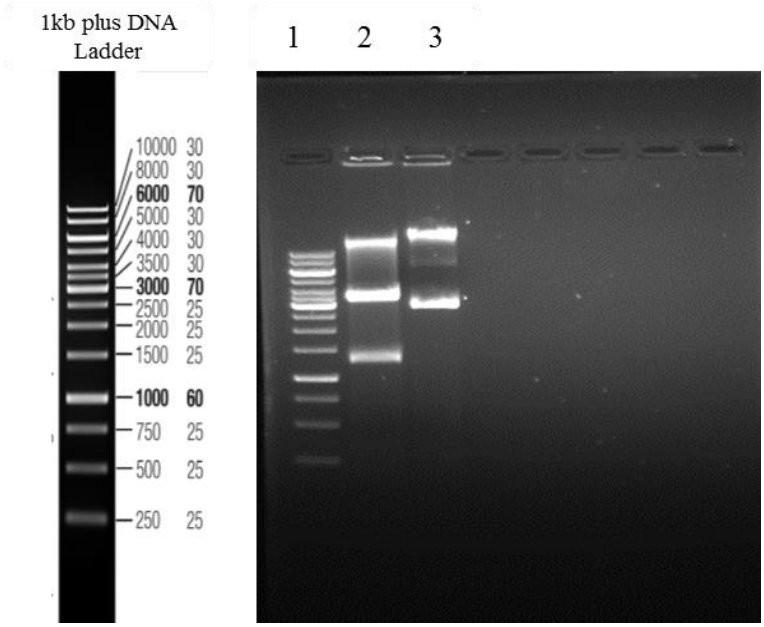


Figure 3-14. The digestion of plasmid pSAM_R1 from the transformed cells.

The Digestion of plasmid pSAM_R1 from the transformed cells. Ladder (1), DNA plasmid prep digested with *Bam*HI and *Xho*I (2), DNA plasmid prep without digestion (3).

3.3.5. Conjugation of *Mc*. *capsulatus* (Bath) and *Ms. trichosporium* OB3b

Experiments and unconjugated controls were set up to transfer pSAM_1 into the methanotraphs *Mc*. *capsulatus* (Bath) and *Ms. trichosporium* OB3b. The cells were plated on NMS agar with 15 and 30 µg/ml final concentration of kanamycin. The cells grew on the plates with and without conjugation for both strains of methanotraph, *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b, which suggested a problem with the antibiotic selection since the unconjugated strain was predicted to grow in the presence of kanamycin. The activity of the kanamycin was checked via using various concentrations of kanamycin (30, 40, 50, 60, 70, 80, 90, 100 µg/ml final concentration) with NMS agar media and methane with both strains of methanotraphs to investigate whether the kanamycin was active or not. After two weeks of incubation with methane, all the plates with different concentration of kanamycin showed growth, whether the cells had been conjugated or not. Results at three representative concentrations are shown in (Figures 3.15, 3.16 and 3.17).

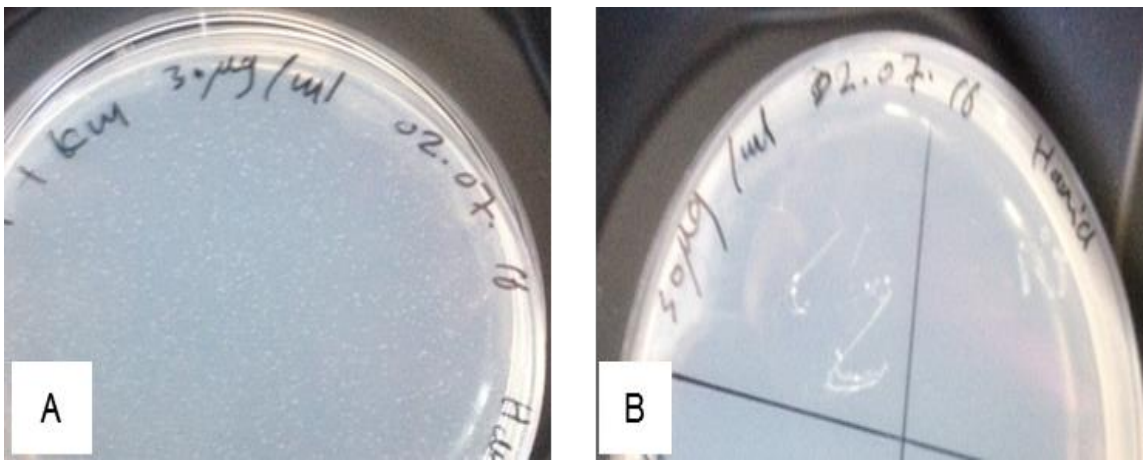


Figure 3-15. *Mc. capsulatus* (Bath) of the conjugation growing with 30 µg/ml kanamycin. *Mc*. *capsulatus* (Bath) (conjugation A & un-conjugated B) growing with 30 µg/ml kanamycin.

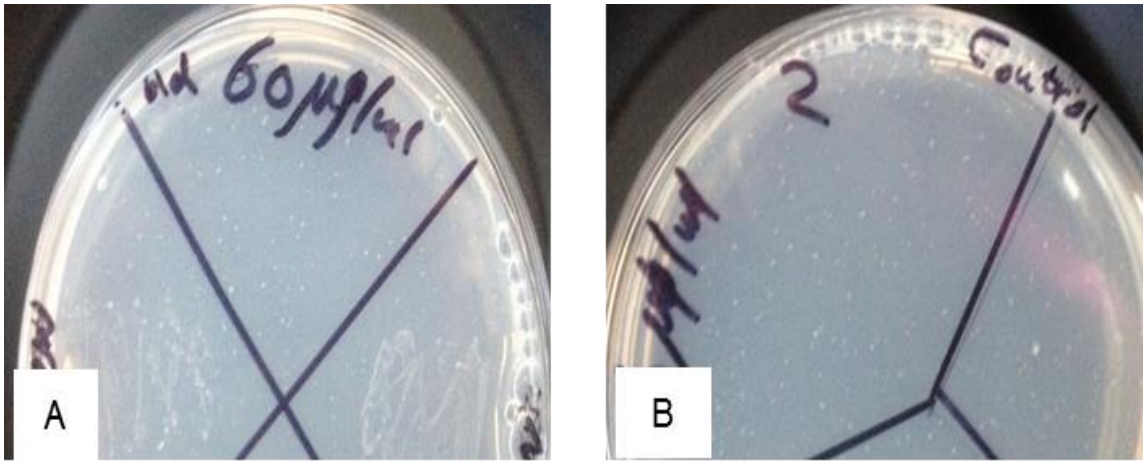


Figure 3-16. *Ms. trichosporium* OB3b growing with 60 µg/ml kanamycin.

Ms. trichosporium OB3b. (conjugation A & unconjugated B) growing with 60 µg/ml kanamycin.

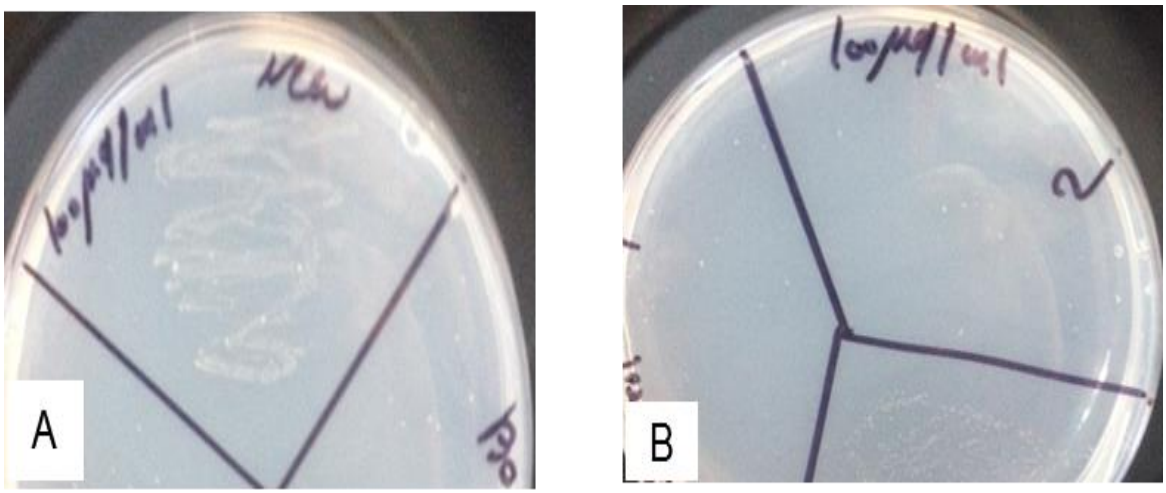


Figure 3-17. *Mc.capsulatus* (Bath) of the conjugation growing with 100 µg/ml kanamycin

Mc. *capsulatus* (Bath) (conjugation A & unconjugated B) growing with 100 µg/ml kanamycin

To investigate further whether the kanamycin was active, the kanamycin sensitive strain *E. coli* JM109 was used. *E. coli* JM109 was plated on LB agar with 50 and 80 µl/ml final concentration of kanamycin with a control (plate without antibiotic). After overnight incubation, the bacteria on the plates with kanamycin had grown both with antibiotic and without. This indicated that the kanamycin was not active under these conditions. For this reason, a new batch of kanamycin was used to test the conjugated cells of *Ms trichosporium* OB3b and *Mc capsulatus* (Bath) and appropriate controls. Two different

concentrations of kanamycin (15 and 30 µg/ml final concentration) were used to grow both strains, *Mc capsulatus* (Bath) and *Ms trichosporium* OB3b in presence of control (wild type) of each strain.

The results showed that the *Ms trichosporium* OB3b cells into which the plasmid pSAM_R1 had been introduced via conjugation grew at both concentrations of kanamycin (Fig. 3.18), presumably due to the resistance gene on the plasmid, while the cells of *Ms trichosporium* OB3b without conjugation (WT) did not grow (Fig 3.19). Approximately 790 colonies were obtained when 100 µl of the conjugated cell suspension was plated on the selective agar. When cells of *Ms trichosporium* OB3b without conjugation were grown on medium without kanamycin, a lawn of bacterial growth was observed, confirming that these cells were viable (Fig. 3.20). These results strongly suggest that the conjugation of pSAM-R1 into *Ms. trichosporium* had been successful.

The strain *Mc capsulatus* (Bath) was conjugated with *E. coli SM10λpir* containing pSAM_R1 and conjugants were plated on NMS medium containing 15 and 30 µg/ml final concentration of the active kanamycin, in parallel with unconjugated controls. None of the plates showed clear colonies of *Mc. capsulatus*. The conjugation plates showed possible contamination (Fig 3.21). The plates without conjugation showed growth of very small of colonies on the plates with 15 µg/ml of kanamycin (Fig 3.22). Because of this result *Mc capsulatus* (Bath) was excluded from the remainder of this study.

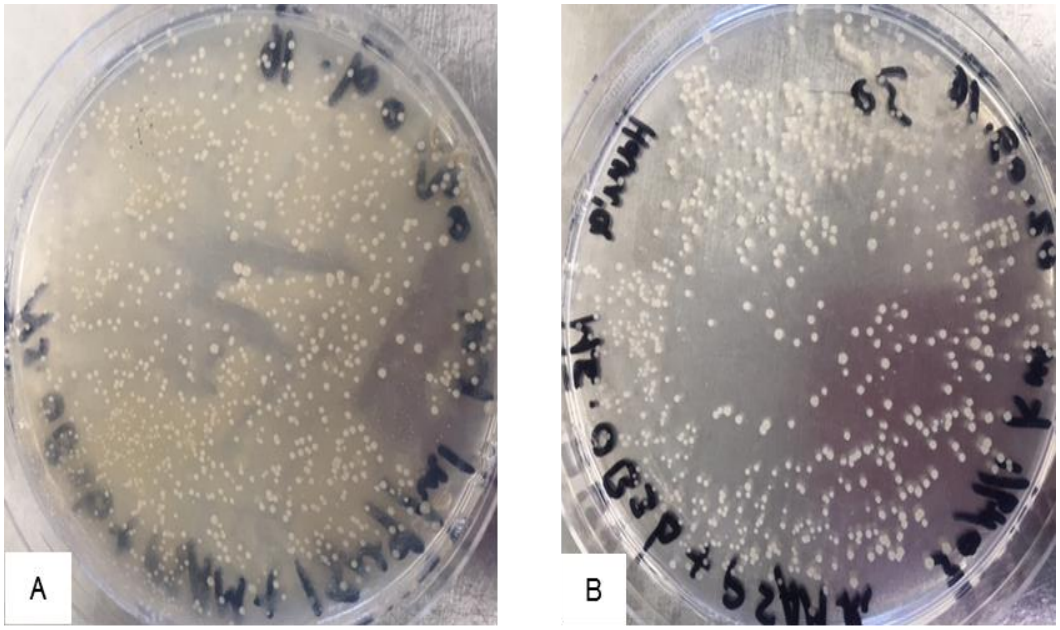


Figure 3-18. Conjugation of *Ms trichosporium* OB3b with 15 µg/ml and 30 µg/ml final concentration of kanamycin.

Conjugation of *Ms trichosporium* OB3b with 15 µg/ml final concentration of kanamycin (A), 30 µg/ml final concentration of kanamycin (B).

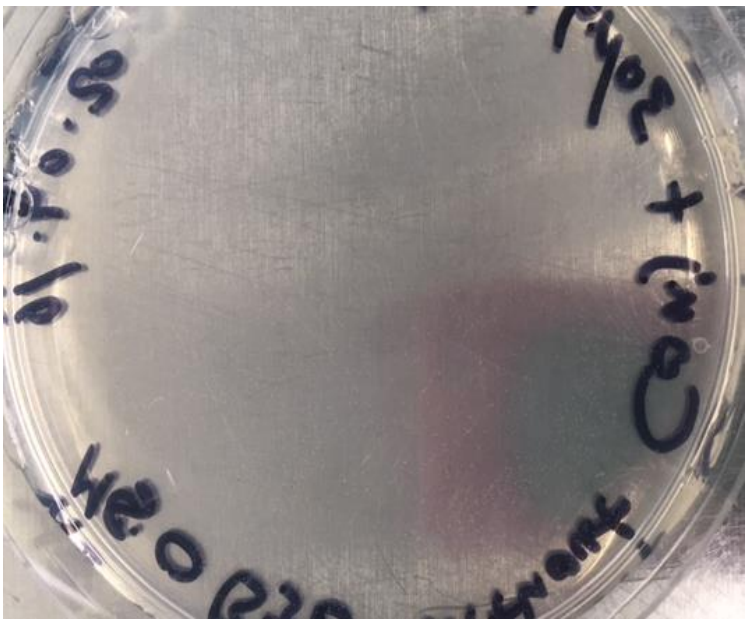


Figure 3-19. *Ms. trichosporium* OB3b without conjugation (WT) plated on NMS medium with 15, 30 µg/ml final concentration of kanamycin.

Ms. trichosporium OB3b without conjugation (WT) did not grow with 15, 30 µg/ml final concentration of kanamycin.

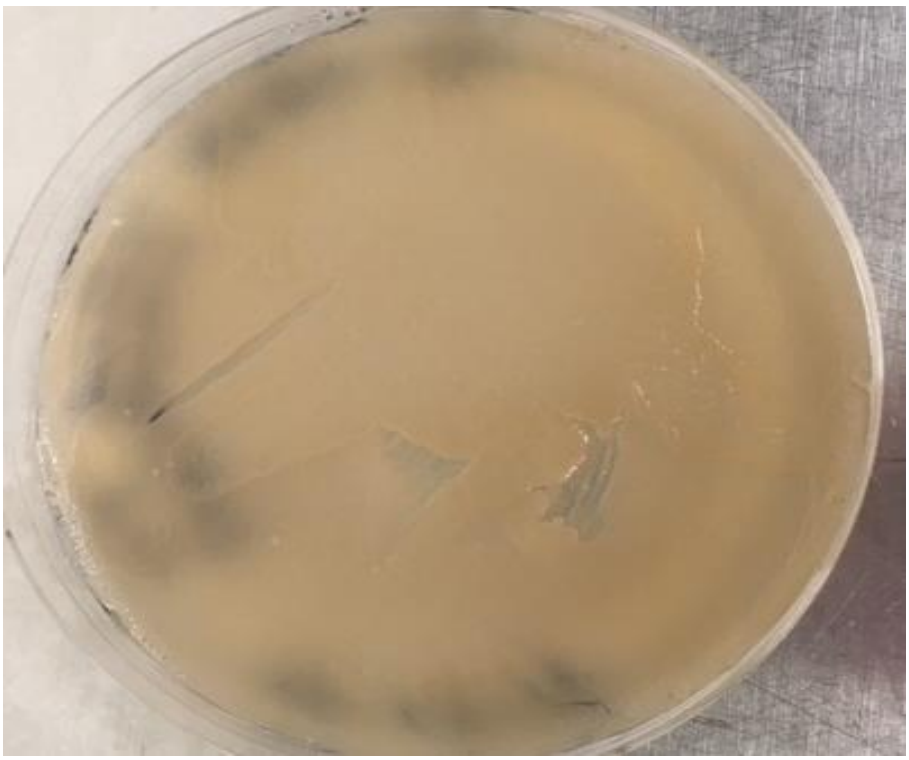


Figure 3-20. *Ms. trichosporium* OB3b without kanamycin and without conjugation

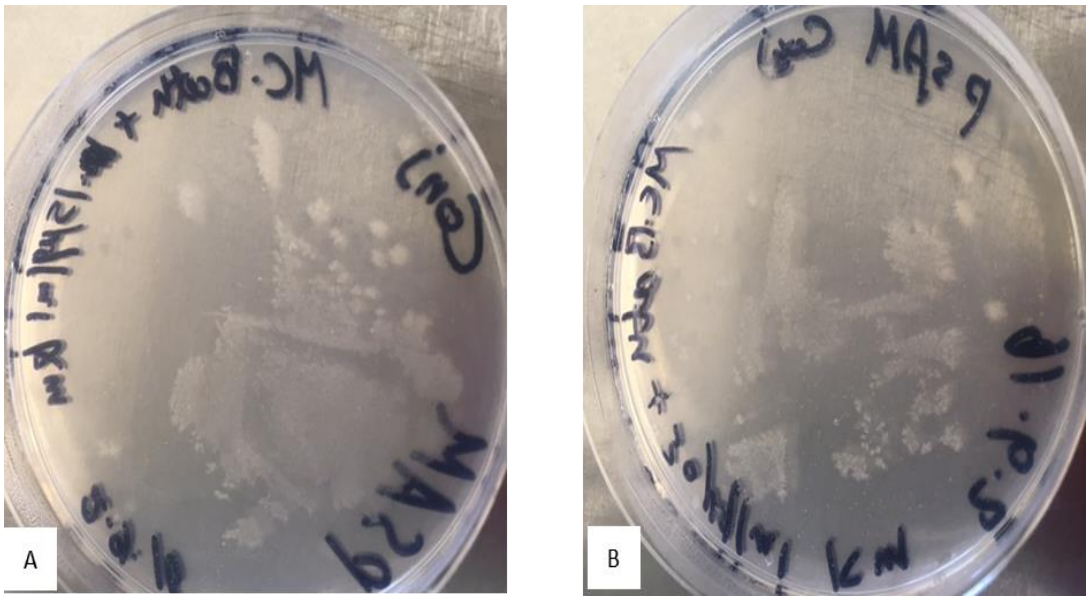


Figure 3-21**.** Conjugation of *Mc capsulatus* (Bath) with 15 µg/ml and 30 µg/ml final concentration of kanamycin.

Conjugation of *Mc capsulatus* (Bath) with 15 µg/ml final concentration of kanamycin (A), 30 µg/ml final concentration of kanamycin (B).

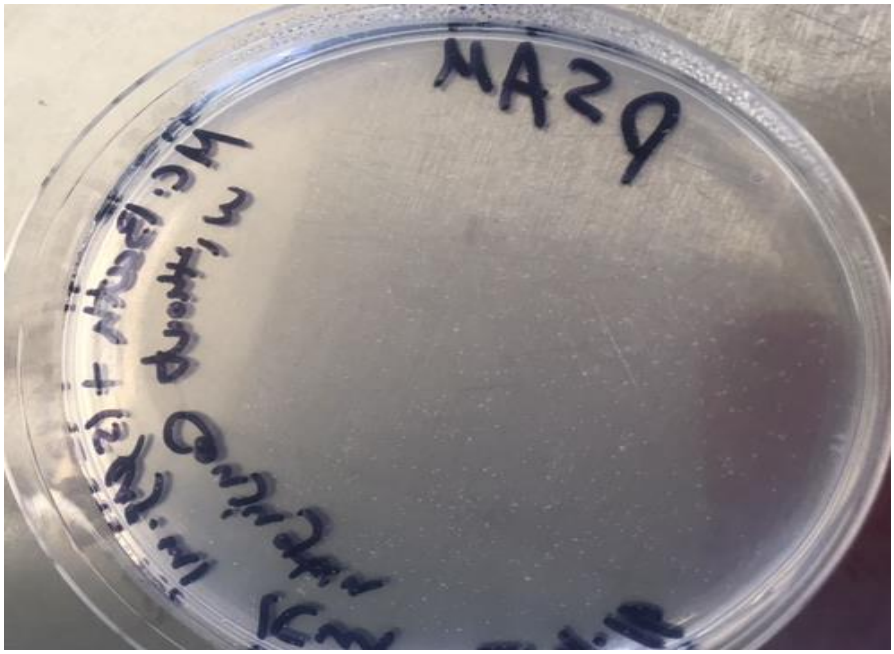


Figure 3-22**.** *Mc capsulatus* (Bath) without conjugation with 15 µg/ml final concentration of kanamycin

3.3.5.1. Testing the resistance of the conjugated cells of *Ms. trichosporium* OB3b with kanamycin and nalidixic acid.

In order to remove the *E. coli* donor strain from the conjugation mixture, it was necessary to use nalidixic acid, to which *E. coli* is sensitive but *Ms. trichosporium* has intrinsic resistance (Lidstrom, Wopat, 1984). When the conjugation was repeated and a loopful of conjugated cell suspension was spread onto NMS medium containing 15 µg/ml final concentration of kanamycin and nalidixic acid (25 µg/ml final concentration), confluent growth was obtained (Fig. 3.23 A). This indicated the feasibility of using the two antibiotics together, kanamycin to select for the plasmid pSAM_R1and nalidixic acid to eliminate any remaining *E. coli* donor cells. The negative control (the parental strain of *Ms. trichosporium* OB3b) did not show any growth on the medium with kanamycin and nalidixic acid (Fig. 3.23B).

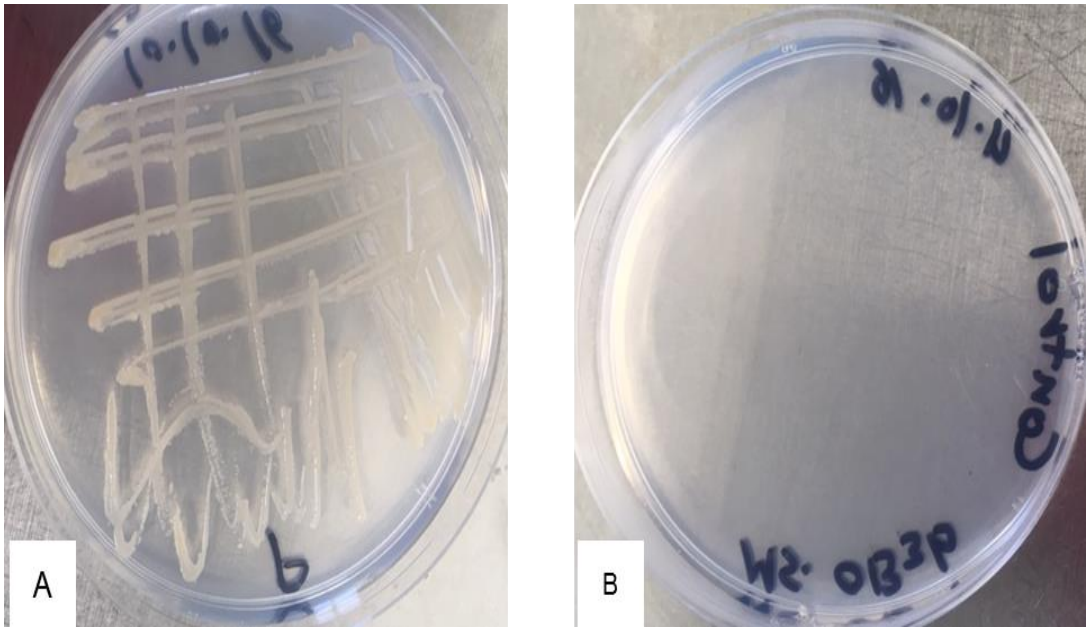


Figure 3-23. *Ms. trichosporium* OB3b after conjugation on NMS agar with kanamycin and nalidixic acid.

(A) *Ms. trichosporium* OB3b after conjugation was grown on NMS agar with kanamycin and nalidixic acid, (B) *Ms. trichosporium* OB3b without conjugation did not grow on NMS with kanamycin and nalidixic acid.

3.4. Discussion

The results in this chapter established conditions for conjugative transfer of the mariner transposon-containing plasmid pSAM_RI from *E. coli SM10λpir to Ms. trichosporium* OB3b. To the author's knowledge no previous study had introduced plasmid pSAM_RI or any other Mariner transposon-containing plasmid into methanotrophs. Because it is difficult to transform plasmids directly into *Ms. trichosporium* OB3b, the plasmid was first electroporated into a suitable strain of *E. coli SM10λpir* and then transferred by conjugation into the methanotroph. *E. coli SM10λpir* was chosen as the *E. coli* strain since it permits replication of pSAM-R1, as well as having the *tra* genes required conjugative transfer.

After troubleshooting the appropriate concentration of kanamycin to use for selection of methotrophs cells that had received the plasmid, each conjugation plate contained approximately 790 colonies of exconjugants (Fig. 3.18). In order to have 87.9 % mutant coverage of the 4,740 coding sequences in the 4,508,832-bp *Ms. trichosporium* OB3b genome, presuming random insertion of the transposon, it would necessary to screen 10,000 colonies. Hence, it appeared that the requisite number of colonies for this coverage could be obtained from around 13 conjugation plates.

Since kanamycin-resistant exconjugants were not obtained from the experiments with *Mc. capsulatus* (Bath), no further attempts to construct a transposon library in this strain were performed.

Restriction analysis confirmed that the plasmid in the donor *E. coli* strain had a restriction pattern consistent with the desired pSAM_R1 (Figs. 3.12-3.14). Further analysis to confirm that the exconjugants had received the pSAM_R1 plasmid and that transposition into the methanotroph chromosome had occurred is detailed in the next chapter.

Chapter 4 Screening and further development of the transposon library

4.1. Introduction

The development of a suitable high-throughput screening or selection system is essential for identifying mutants with the desired properties in any kind of random or quasi-random mutant library (Bharani *et al.*, 2015; Zhao, Arnold, 1997).

The main aim of this study was to discover the genes that are responsible to reduce the activity of the selenite in the *Ms*. *trichosporium* OB3b. In the previous chapter the plasmid pSAM-R1 containing the transposon was transferred successfully to *Ms*. *trichosporium* OB3b from E. *coli SM10 λ pir* via conjugation process. This chapter investigated whether the transposon was transferred from the plasmid to the chromosomal DNA of *Ms*. *trichosporium* OB3b by three different methods which were PCR, Southern blot hybridization and sequencing. The work then went on to screen for changes in the reduction of selenite to elemental selenium, which was a reaction well suited to high-throughput screening since the product of the reaction is red in colour and can be easily detected by eye in the colonies of strains of microorganisms grown as colonies on agar plates.

4.2. Materials and Method

4.2.1. Polymerase Chain Reaction (PCR)

To find out whether the fragment of the transposon from the plasmid pSAM_R1 was incorporated into the putative *Ms. trichosporium* exconjugants, a polymerase chain reaction (PCR) was used to amplify the fragment of sequence of plasmid pSAM-R1. A 125 µl of master-mix was mixed with primers 10 µl of TrF, 10 µl of TrR (Table 4.1) and 85 µl of sterile deionised water. The total volume of the solution became 230 µl, and then 23 µl of the resulting mix then was added to 2µl of DNA template sample with concentration approximately 50 ng of chromosome DNA. Standard PCR cycling conditions consisted of 94ºC for 2 min, followed by 35 cycles of 94ºC for 15 seconds, 48ºC for 30 seconds and 72ºC for 1 min followed by a final extension step of 72ºC for 4 min. PCR amplified product (20 µl) was mixed with 5 µl of loading dye, and then the PCR amplified product was electrophoresed (carried out in 1 x TBE buffer at 100V for 45 minutes) in a 0.9% agarose gel with 0.2 µg/ml ethidium bromide alongside DNA molecular markers and visualized under UV illuminator.

Table 4-1. Oligonucleotide primers

Primer name	Primer sequence 5'-3'	Reference
TrF	TAATACGACTCACTATAGGG	(Lampe *et al*., 1999)
TrR	CGATTTAGGTGACACTATAG	

4.2.2. Southern blot hybridization

To confirm the insertion of the selected transposon into the chromosome of the *Ms. trichosporium* OB3b mutant, Southern blot hybridisation was investigated. The first step was digestion of *Ms*. *trichosporium* OB3b DNA.

4.2.2.1. Genomic DNA isolation from *Ms*. *trichosporium* OB3b

Two kits were used for purification of genomic DNA from *Ms. trichosporium* OB3b strain. Initially the Qiagen DNA purification kit was used as detailed in the results chapters. This yielded DNA that did not cut with restriction enzymes, so an alternative kit, the Qiagen genomic tip 20 kit, was used. Both kits were used according to the manufactures' instructions.

4.2.2.2. Genomic DNA isolation from *Ms. trichosporium* OB3b ex-conjugants using Promega Purification of DNA extraction

The culture of ex-conjugants (*Ms*. *trichosporium* OB3b with plasmid pSAM_R1) was used to prepare the DNA.

After five days of incubation of the culture, 1 ml of culture was pipetted into a 1.5 ml microcentrifuge tube and centrifuged at 13,000-16,000 × *g* for 2 minutes to pellet the cells. 600 µl of lysis solution was added after removal of the supernatant. The contents of the tube were gently pipetted up and down until the cells were re-suspended and the mixture was then incubated at 80°C for 5 minutes to lyse the cells. The suspension was then cooled to room temperature. To the cell lysate, 3µl of RNase solution from the kit were added and the tube inverted 2-5 times to mix. The tubes were then incubated at 37°C for 15-60 minutes. The suspension was cooled to room temperature. 200µl of Protein Precipitation Solution was added to the RNase-treated cell lysate and vigorously vortexed at high speed for 20 seconds to mix Protein Precipitation Solution with the cell lysate. The cell lysate suspension was then incubated on ice for 5 minutes and centrifuged at 13,000-16,000 × *g* for 3 minutes. The supernatant containing the DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600µl of isopropanol at room temperature. The suspension was gently mixed by inversion until the thread-like strands of DNA formed a visible mass which was centrifuged at 13,000-16,000 × *g* for 2 minutes. The supernatant was carefully poured off and the tube drained on clean absorbent paper. To the DNA pellet, 600µl 70 % (vol/vol) ethanol was added at room temperature and the tube was gently inverted several times to wash the DNA pellet. The suspension was centrifuged at 13,000-16,000 × *g*

for 2 minutes and the ethanol carefully aspirated. The DNA pellet in the tube was drained on clean absorbent paper and allowed to air-dry for 10-15 minutes. To the tube 100µl of DNA Rehydration Solution was added and the DNA rehydrated by at 65°C for 1 hour. Periodically, the solution was gently mixed by tapping the tube. The concentration of each sample was quantified by using the Nano-drop spectrophotometer, then the DNA was stored at -20°C. To ascertain the presence of the DNA gel electrophoresis was performed.

4.2.2.3. Genomic DNA isolation from Ms. trichosporium OB3b exconjugants using Qiagen genomic-tip 20/ kit

A 50 ml of cultures of ex-conjugants (*Ms. trichosporium*OB3b with plasmid PSAM_R1) was used to prepare the DNA. Six Eppendorf tubes each containing 1 ml liquid culture of one of the ex-conjugants ($ODA_{600:0.5\text{-}1}$) (Table 6) were centrifuged at 5000 x *g* for 5 min to pellet the cells. Six Eppendorf tubes, each containing 1 ml liquid culture of one of the ex-conjugant *Ms. trichosporium* OB3b strains (OD A_{600} 0.5-1) were centrifuged at 5000 x *g* for 5 min to pellet the cells. The pellets were resuspended in 1 ml of buffer B1 by vortexing and 20 µl lysozyme (100mg ml^{-1}) and 45 µl of proteinase K solution (20 mg ml^{-1}) were added. The reaction mix was incubated at 37°C for 30 min, 0.35 ml of buffer B2 added and the mixture was incubated at 50°C for 30 min. A genomic tip 20 column was equilibrated with 2 ml of QBT buffer (supplied in a QIAGEN Maxi preparation Kit) and allowed to flow through by gravity.

The lysed cell-containing sample was added to the column and washed with 3 x 1ml Qiagen QC buffer. The genomic DNA was eluted using 2 x 1ml Qiagen QF buffer and precipitated by adding 1.4ml isopropanol at room temperature. The tubes were centrifuged at 8000 x *g* for 20 min at 4°C to pellet the DNA. The pellet was washed with 70% Ethanol, centrifuged at 8000 x g for 10 min at 4°C and allowed to air-dry for 10 min. The pellet was resuspended in 50-100µl mM Tris-HCl (pH 8.5) and placed in a shaker at 30°C overnight to dissolve the DNA (or 55°C for 1 - 2 hours). The concentration of each sample was recorded by using the Nano-drop spectrophotometer, then the DNA was

stored at -20°C. Buffers B1, B2, QBT, QC and QF were prepared according to the manufacturer's instructions (appendix). The proteinase K solution (20 mg ml^{-1}) was supplied by Qiagen.

4.2.2.4. Digestion of chromosomal DNA samples from *Ms. trichosporium* OB3b ex-conjugants

Ten samples of DNA of *Ms. trichosporium* OB3b were digested with two different restriction enzymes (*Bam*HI, and *Hin*dIII). (Table 4.2) contains the reaction components and the total of reaction mixture was 15 µl. The volume of *Ms. trichosporium OB3b* DNA solution depended on the concentration of the DNA of *Ms. trichosporium OB3b*. The reaction mixture was incubated at 37ºC for two hours and overnight. The DNA fragments digested by restriction enzymes were separated on a 0.9% agarose gel with 0.2µl/ml ethidium bromide and visualized under ultra violet (UV) light.

Table 4-2. Reaction components of digestion DNA of *Ms. trichosporium* OB3b ex-conjugants

Samples	**Concentration of the DNA *Ms. trichosporium* OB3b µg/ml**	***Restriction enzymes *Bam*HI or *Hin*dIII µl**	**DNA solution of *Ms. trichosporium* OB3b µl**	***Restriction enzyme buffer µl**	**Sterile water** µl
1	370.8	1	5.4	1.5	7.1
2	150.5	1	12.2	1.5	0.3
3	170.2	1	11.7	1.5	0.8
4	140.5	1	12.5	1.5	Zero
5	83.2	1	12.5	1.5	Zero
6	253.3	1	7.9	1.5	4.6
7	474.5	1	4.2	1.5	8.3
8	133.3	1	12.5	1.5	Zero
9	307.2	1	6.2	1.5	6
10	53.5	1	12.5 l	1.5	Zero

* Each restriction enzyme was used separate experiment.

4.2.5. Preparation of DNA samples for sequencing

The samples of *Ms. trichosporium* OB3b putative transposon mutants that were believed to contain plasmid pSAM_R1 were grown on the NMS agar with kanamycin (15 µg/ml final concentration), and *Ms. trichosporium* OB3b (WT) as a control on the separated plate in the presence of methane and incubated at 30°C. After two weeks of growth, the bacteria were harvested from the plates, and transferred into fresh sample carriage tubes supplied by Mictobes NG, containing glycerol and porous beads. The samples were sent to the Microbes NG team in the University of Birmingham for DNA sequencing.

DNA extraction, sequencing and preliminary bioinformatic assembly and manipulation of the sequence were performed by Microbes NG as detailed in the remainder of this section. The information below is taken from the Microbes NG website (https://microbesng.uk/microbesng-faq/).

Three of the beads containing the microbial culture were washed with extraction buffer containing lysozyme and RNase A, incubated for 25 min at 37°C. Proteinase K and RNaseA were added and incubated for 5 min at 65°C. Genomic DNA was purified using an equal volume of SPRI beads and resuspended in EB buffer. DNA was quantified in triplicate with the Quantit dsDNA HS assay in an Ependorff AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: two nanograms of DNA instead of one were used as input, and PCR elongation time was increased to 1 min from 30 seconds. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on the Illumina HiSeq using a 250bp paired end protocol.
Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 [1]. De novo assembly was performed on samples using SPAdes version 3.7 [2], and contigs were annotated using Prokka 1.11 [3].

4.2.5.1. Sequences data analysis

The draft genomes were received as FASTA files. To detect the transposon position and the number of inserted DNA segments, each file was uploaded to the Mauve sequence analysis (Rissman *et al.*, 2009) for alignment against the reference genome (*Ms. trichosporium* OB3b wild type; ENA databank accession number CP023737).

To define the position of inserted transposon in each contig (assigned as numbered Nodes by the Mauve genome assembly function), the selected draft genome was blasted against the transposon sequences to find the location of the transposon sequence.

4.2.5.2. Screening of transposon libraries

The library of mutants inactivated in different genes was screened to identify mutatnts which were deficient in the remediation reactions, and hence identify genes that are involved. Single colonies of the ex-conjugants were picked from the plates after conjugation using sterile toothpicks and transferred into a grid arrangement on fresh NMS agar plates which had been marked out by Microplate Replicators (supplied in Boekel Scientific) on the NMS agar with 15 µg/ml final concentration of kanamycin. The plates were incubated at 30°C for 2-4 weeks in the presence of methane, then the colonies were transferred to 96-well microtiter plates in which each well contains 100 µl of NMS with 30% glycerol

4.2.6. Calculation of the numbers of colonies required for screening the library

Each conjugation plate contained approximately 790 colonies of exconjugants as detailed in the previous chapter (Fig. 3.16). In order to have 87.9 % mutant

coverage of the 4,740 coding sequences in the 4,508,832-bp *Ms. trichosporium* OB3b genome, presuming random insertion of the transposon, it would necessary to screen 10,000 colonies. Hence, it appeared that the requisite number of colonies for this coverage could be obtained from around 13 conjugation plates. This estimate was obtained by using the equation $N = \ln(1-P)/\ln(1-f)$, where **P** is the probability of the library including any particular gene, **f** is the fractional proportion of the genome in a single gene (Sambrook, *et al* 1989)

Here 4,740/4,508,832) and **N** is the number of recombinants to be screened (here 10,000).

4.2.7. Analysis of putative selenite non-reducing mutant using NMS solid media

The mutant colonies that were unable to reduce the selenite were investigated further. White colonies from plates of the transposon library were re-grown on NMS agar with 15 µg/ml final concentrations of kanamycin and 10 µg/ml Se as selenite. A kanamycin-resistant clone from the transposon library that gave red colonies on selenite-containing medium was used as the positive control. The plates were incubated in 30°C for 2 weeks in the presence of methane.

4.2.7.1. Effect of selenite on the mutants in the NMS liquid media

As detailed below, the test of selenite reduction sometimes gave an unclear result, which was attributed at least in part to the colour of the agar medium and the effect of kanamycin retarding the growth of some of the strains. To obtain clearer results, tests of selenite reduction were conducted in liquid media. Three white colonies of the re-streaked putative selenite non-reducing mutant were transferred to three flasks containing 50 mL of NMS without kanamycin. When the OD_{600} of the cultures reached 0.5 - 0.7, a sterile sodium selenite stock solution was added to give 10 µg/mL of Se as selenite. Two controls were set up in triplate and treated in exactly the same way. These controls were: (1) a mutant clone from the transposon library that gave red

colonies on selenite-containing plates and (2) wild type colonies of *Ms. trichosporium* OB3b. The flasks were incubated at 30°C in the presence of methane for 10 days and any changes in the colour of the medium were recorded.

4.2.7.2. Effect of selenite and kanamycin on the mutants in the NMS liquid media

To determine the effect of selenite and kanamycin on mutants in liquid NMS media, single colonies of mutants (white and red) were re-grown separately in triplicate in flasks with NMS liquid media (50 ml) with kanamycin (15 µg/ml final concentration) and methane. Selenite was added to each flask (to give a concentration of Se of 10 µg/mL) when the OD_{600} reached 0.5 - 0.7. Then the flasks were incubated at 30°C with the presence of methane and any changes in colour were recorded.

4.2.8. Effect of sodium formate on the white mutant

In order further to characterise the mutant that appeared to be impaired in the selenite reducing reaction, the hypothesis was Is the selenite reduction could be resorted by adding the electron donor sodium formate?, the hypothesis was investigated. The test strain was grown in triplicate in NMS liquid media without kanamycin until the OD_{600} reached 0.5 - 0.7. Selenite and formate were added to each experiment as given in (Table 4.3). The experiment was done in triplicate.

Table 4-3. Contents of each flask in the experiment with formate

Test number	**Amount and interaction components (three 250-mL flasks with 50 ml NMS for each test), (concentration of Se 10 µg/mL)**		
Test 1	White mutant	500 µl Na $(SeO_3)^{2-}$	
Test 2	White mutant	500 µl Na $(SeO_3)^{2-}$	5 mM HCOONa
Test 3	Wild type	500 µl Na $(SeO_3)^{2-}$	
Test 4	Wild type	500 µl Na $(SeO_3)^{2-}$	5 mM HCOONa
Test 5	NMS media	500 µl Na $(SeO_3)^{2-}$	5 mM HCOONa

4.3. Results

4. 3.1. Polymerase Chain Reaction (PCR)

With the intention of testing the presence of the transposon in the *Methylosinus trichosporium* OB3b ex-conjugants' genomes, PCR with primers TrF and TrR (Table 4.1) was used. The results showed no products from the PCR using the chromosomal DNA from the ex-conjugants, although the positive control using purified pSAM_R1 DNA gave a clear product of about 3146 bp (Fig 4.1 A). The primers flanked the complete sequences of the transposon as shown in (Fig 4.1 B) and are able to amplify this sequence as indicated by the positive control PCR using the pSAM_R1 plasmid as the template. More careful analysis of the structure of the plasmid (Fig 4.1B) showed that one of the primers used in the PCR binds outside the region of the transposon that was predicted to insert into the chromosome (flanked by the two inverted repeat regions shown in red in (Fig 4.1B); Perry and Yost, 2015). Hence this result is consistent with the insertion of the transposon into the chromosome, though additional evidence was needed for the presence of the transposon within the chromosome of these putative transposon mutants.

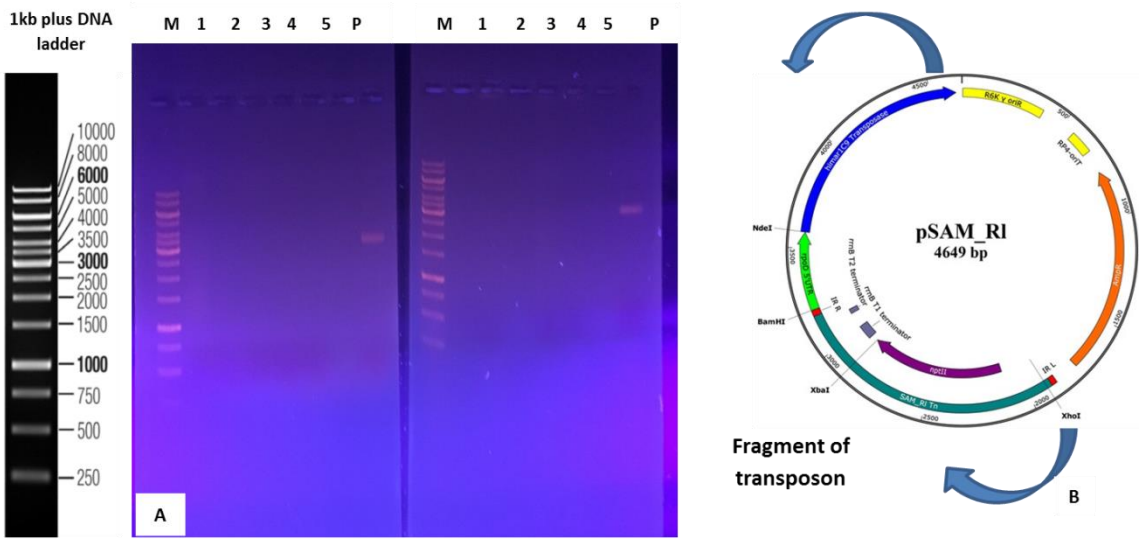


Figure 4-1. Agarose gel of the PCRs using primers TrF and TrR.

Tracks: ladder (M), PCRs from the exconjugants (samples 1-10), PCR with plasmid pSAM_R1 as the template (P). Panel B shows the map of plasmid pSAM_R1; the arrows around the outside of the plasmid show the fragment of the plasmid amplified by primers TrF and TrR

4.3.2. Southern blot hybridization

With the intention of determining the sites of transposon insertion in the chromosome of the putative mutants, Southern blot hybridisation was investigated. The Promega kit was used for DNA extraction of the chromosomal DNA from the putative mutants.

In order to be able to perform Southern blotting it was necessary to cut the DNA with a restriction enzyme that would allow the blot to show the different location of the transposon in the chromosome of the ex-conjugants. *Bam* HI was used to digest the purified DNA. When the digested chromosomal DNA was analysed by means of agarose gel electrophoresis in parallel with a positive control of pSAM_R1 digested with the same enzyme (Fig. 4.2), it was clear that the chromosomal DNA had not been digested although the control showed that the enzyme was active.

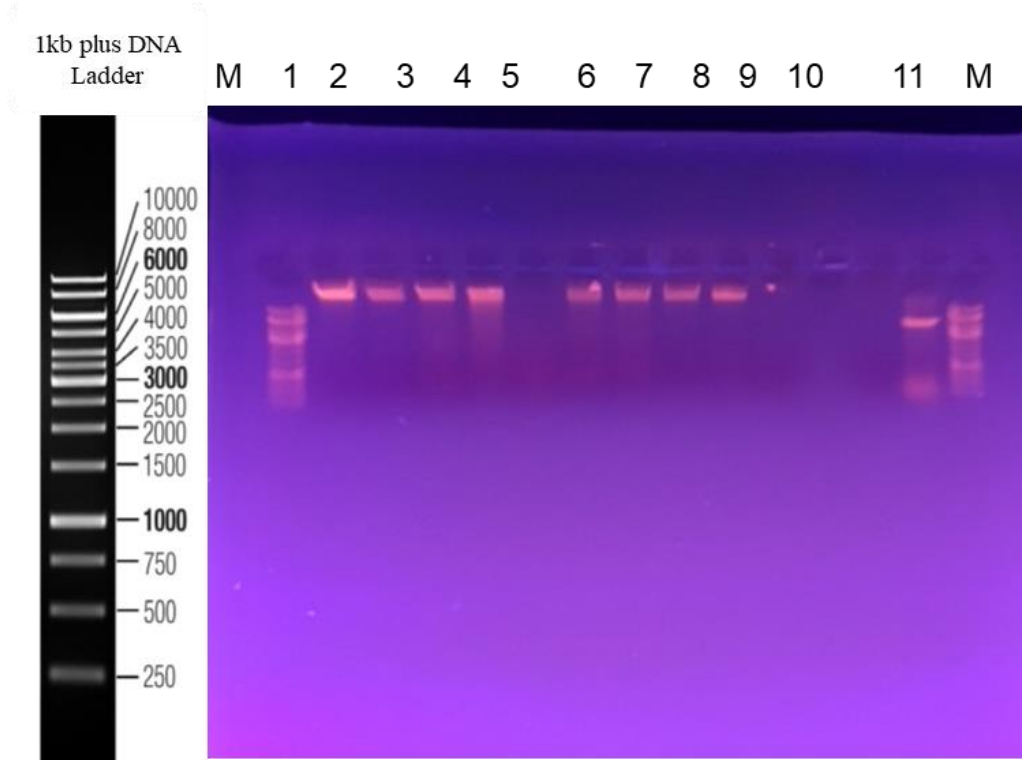


Figure 4-2. Digestion of DNA from *Ms. trichosporium* OB3b ex-conjugants with *Bam* HI.

Ladder (M); 1 to 10, samples of DNA from *Ms. trichosporium* OB3b ex-conjugants digested with *Bam* HI, Track 11 contains plasmid pSAM_R1 DNA digested with the same enzyme.

The chromosomal DNA of the putative mutants was purified again using a different kit (the Qiagen genomic-tip 20/ kit) with the intention of preparing purer and higher-concentration DNA. DNA purified in this way from three mutants was then digested with *Hin*dIII (Fig. 4.3). As previously, there was no evidence of digestion of the chromosomal DNA. The digestion of the DNA from the mutants was repeated several times with different enzymes such as *Xho*I, *Rsa*I, but the results still gave no evidence of digestion of the DNA of mutants on the gel. Rather than spend further time troubleshooting the restriction digests and Southern blotting technique, which would give only partial information about the insertion sites of the transposon, it was decided to perform whole genome sequencing instead. This, it was hoped, would not only confirm the presence of the transposons but also indicate their exact location.

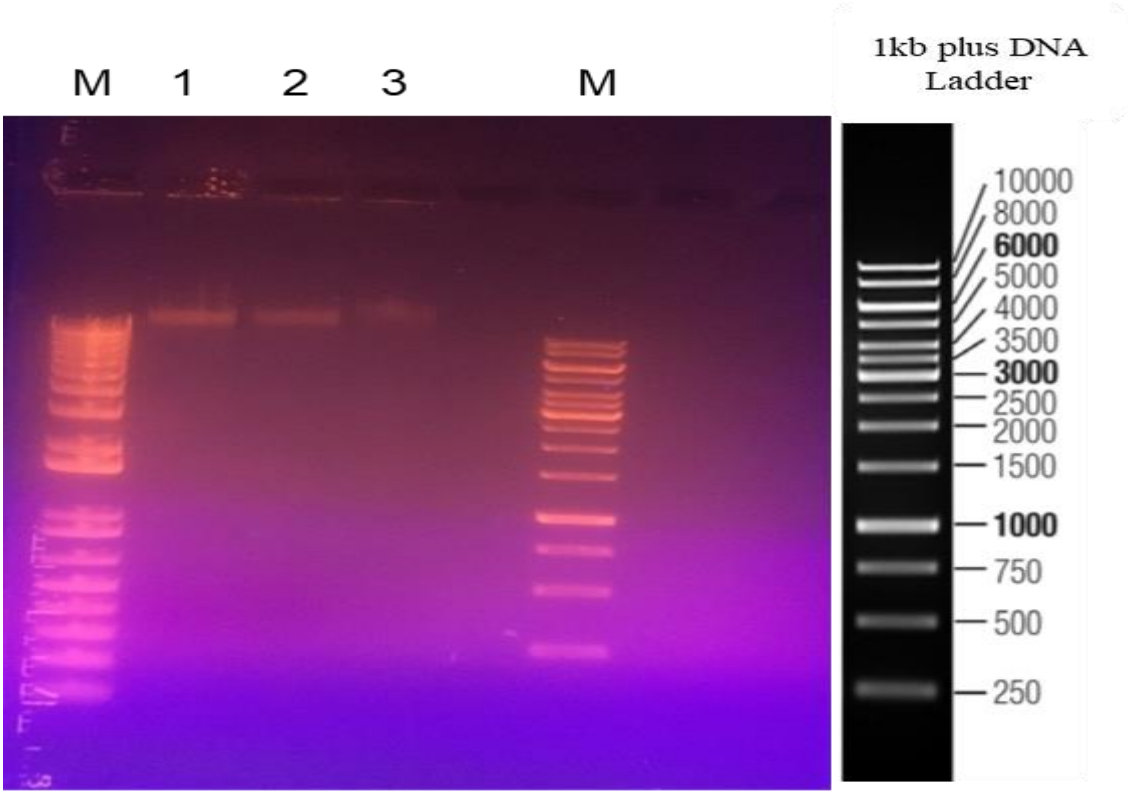


Figure 4-3. Digestion of DNA from *Ms. trichosporium* OB3b ex-conjugants with *Hin*d3. Ladder (M), 1 to 3 samples of *MS.* OB3b conjugation with digest by *Hin*d3.

4.3.3. Whole genome sequencing

Cultures of the putative transposon mutants of *Ms*. *trichosporium* OB3b were prepared for submission for whole-genome sequencing to check the position of the transposon in the genome. Eleven samples were prepared: ten putative transposon mutants and one sample of wild-type of *Ms*. *trichosporium* OB3b, as the parental strain. Cells of these clones were prepared and sent for genome sequencing to Microbes NG as detailed in section 4.2.5. The tubes were barcoded from 9418 to 9428 (Table 4.3). DNA extraction, sequencing and assembly of sequence reads into contigs were performed by Microbes NG. The wild-type *Ms. trichosporium* OB3b strain and nine of the putative mutants gave good enough sequence data to permit analysis. The sample 9418 did not yield useful sequence data.

Table 4-4. Description of samples for sequencing

Sample	**Barcode of the first conjugation samples provided to Microbes NG**	**Type of bacterial sample**
1	9419	*Ms. trichosporium* OB3b with conjugation
2	9420	*Ms. trichosporium* OB3b with conjugation
3	9421	*Ms. trichosporium* OB3b with conjugation
4	9422	*Ms. trichosporium* OB3b with conjugation
5	9423	*Ms. trichosporium* OB3b with conjugation
6	9424	*Ms. trichosporium* OB3b with conjugation
7	9425	*Ms. trichosporium* OB3b with conjugation
8	9426	*Ms. trichosporium* OB3b with conjugation
9	9427	*Ms. trichosporium* OB3b with conjugation
10	9428	*Ms. trichosporium* OB3b (WT) (Reference)
Sample	**Barcode of the second conjugation samples provided to Microbes NG**	**Type of bacterial sample**
11	14741	Mutant of *Ms. trichosporium* OB3b
12	14742	Mutant of *MS. trichosporium* OB3b
13	14743	Mutant of *Ms. trichosporium* OB3b
14	14744	Mutant of *MS. trichosporium* OB3b
15	14745	Mutant of *Ms. trichosporium* OB3b
16	14746	Mutant of *Ms. trichosporium* OB3b
17	14747	Mutant of *Ms. trichosporium* OB3b
18	14748	Mutant of *Ms. trichosporium* OB3b
19	14749	Mutant of *Ms. trichosporium* OB3b
20	14750	Mutant of *Ms. trichosporium* OB3b (White colony)

4.3.3.1. Multiple genome alignment

Mauve Contig Mover (MCM) was employed to re-order contigs for each new draft genome based on comparison to *Ms. trichosporium* OB3b genome (Rissman *et al*., 2009) from the ENA databank (accession number CP023737). This independent draft genome re-ordering was the first step towards comparing the new draft genome with the reference genome Fig 4. 4. Multiple genome sequence alignments (pairwise alignments between the reference and each mutant) were generated using Progressive Mauve on the reordered contigs. These pairwise genome alignments indicated the presence of large homologous regions shared across the genomes compared. However, there was a region within the genomes from the putative transoposon mutants that displayed no similarity to the reference genome. The additional segments were conserved among all nine putative mutant draft genomes but were not present in the genome data obtained in parallel for the wild-type *Ms. trichosporium* OB3b strain. Blast searches of the additional DNA present in the chromosomes of the putative transposon mutants were performed. These searches confirmed that the extra DNA was the Mariner transposon, and the percentage identity to the transposon sequence in the pSAM_R1 plasmid (C. Yost, personal communication) was 99% for all the putative mutant samples (Fig 4.5).

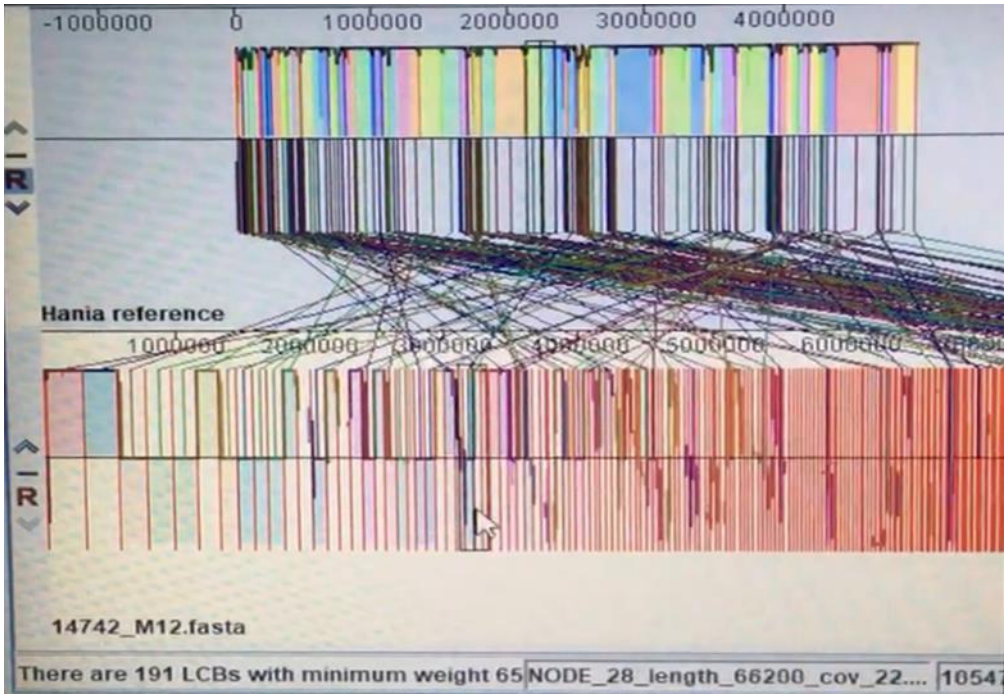


Figure 4-4 Mauve to comparing between the new draft genome with the reference (wild-type *Ms. trichosporium* OB3b strain)

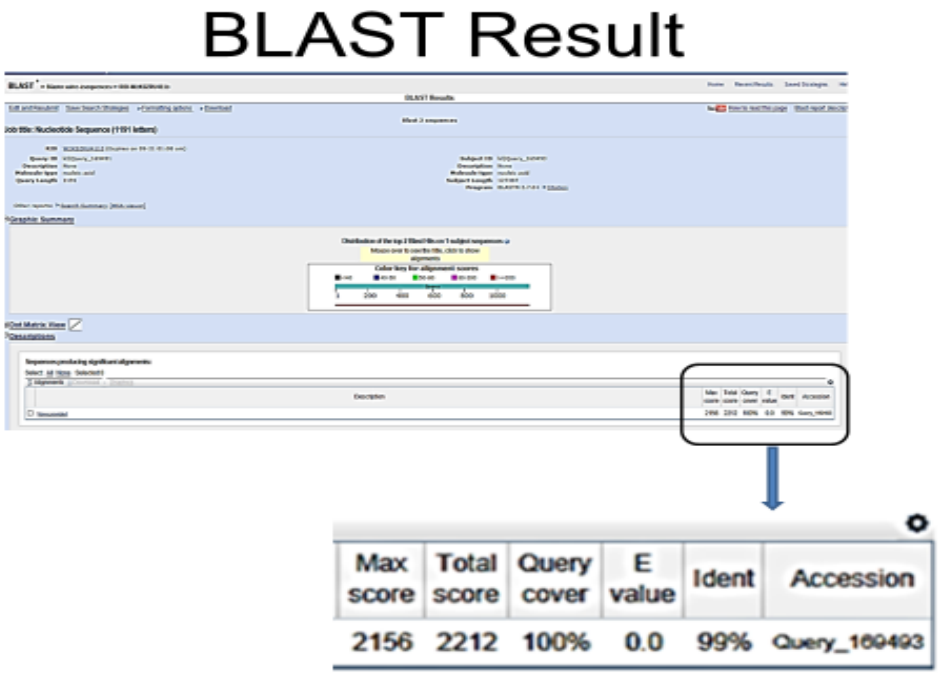


Figure 4-5. Result from BLAST programme with percentage insertion of transposon in the chromosome of *Ms. trichosporium* OB3b

4.3.3.2. Genome sequencing of the *Ms. trichosporium* OB3 mutants and refinement of the transposon mutagenesis protocol

In the first transposon mutant library constructed, out of eight mutants sequenced, only 3 insertion sites were observed (Fig. 4.6 and Table 4.4). This suggested either “hot-spotting” of the transposon or clonal amplification so that a small number of progeny clones were overrepresented in the sample sequenced. If clonal amplification were the issue, it was reasoned that the problem could be reduced by reducing the time of incubation of the methanotroph cells with the donor *E. coli* strain, to minimize the multiplication of the exconjugants before they were plated to obtain single colonies. The conjugation protocol described in Chapter 3 was therefore modified to investigate shorter conjugation times of 8 and 16 h, rather than the 24 h used previously. Conjugation for 8 and 16 h yielded only very small colonies (Fig 4.6) that may have been donor cells or methanotrophs that had not received the plasmid. In contrast, conjugation for 24 h yielded many large colonies. The new transposon library from the 24 h conjugation period was therefore used in all further experiments.

Subsequently out of 10 clones sequenced from the new transposon library (24 h conjugation time) all were unique mutations that were distributed around the genome (Fig. 4.7 and Table 4.4). The tubes used to obtain these genome sequences were barcoded from 14741-14749 (Table 4.3). The second transposon library was used for screening to detect mutants deficient in the reduction of selenite to elemental selenium as detailed below.

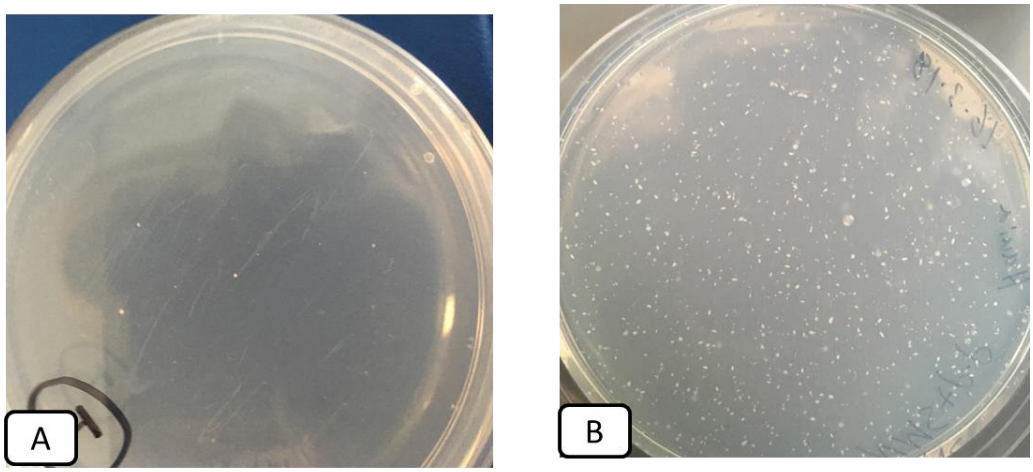


Figure 4-6. Growth of exconjugants of *Ms. trichosporium* OB3b

Contain 15 µg/ml final concentrations with different incubation. 8 hours (A), 16 hours (B)

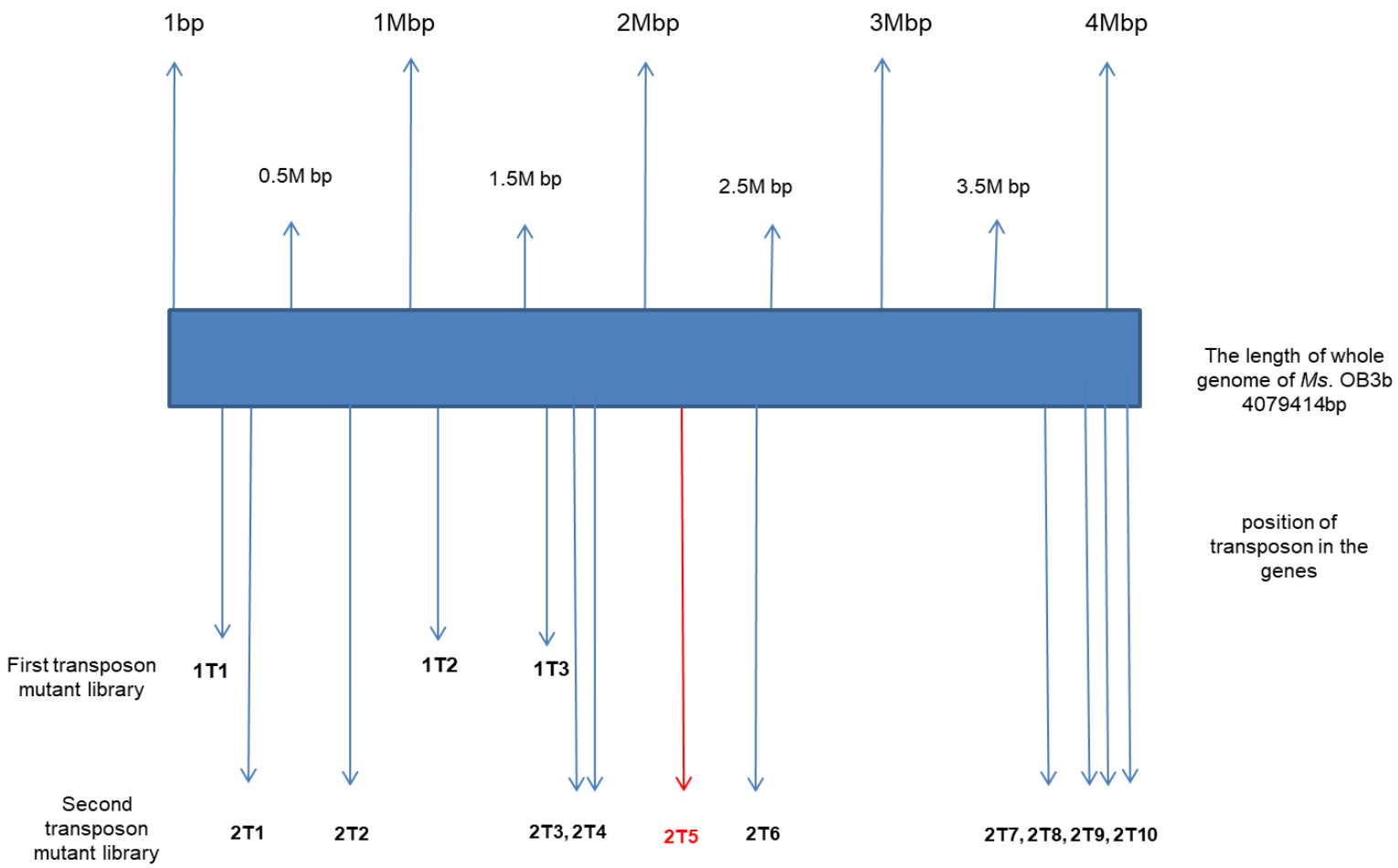


Figure 4-7. Position of the gene which responsible to reduce the selenite.

The blue arrow shows the positions of transposon insertions in the genome of *Ms. trichosporium* OB3b. The red arrow (2T5) show the position of the gene which responsible to reduce the selenite.

Table 4-5. Locations of transposon insertions in the mutants.

Clone	Position of Transposon in the genes (bp)	Position of the gene in genome (bp)	Locus _tag	Gene interrupted
Sample from first transposon library				
1T1 (sample 1*, 4, 9)	120297-12299	120204..121505	CQW49_00600	Histidinoldehydrogenase
1T2 (sample 6, 7, 8)	1251870-1251871	1249265..1252692	CQW49_05935	histidine kinase
1T3 (sample3,5)	1613343-1613344	1611758..1613428	CQW49_07815	phage portal protein
Sample from second transposon library				
2T1 (sample 11)	1640674-1640675	1640581..1642134	Within a CRISPR family repeat region	
2T2 (sample 15)	343850-343851	343299..345140	CQW49_01680	methyl-acceptingchemotaxisprotein
2T3 (sample 14)	773653-773655	773242..773598	CQW49_03655	hypothetical protein
2T4 (sample 19)	1784643-1784650	1784187..1785494	CQW49_08640	FAD-dependent oxidoreductase
2T6 (sample 17)	2412386-2412387	2412157..2413719	CQW49_11630	cytochrome d terminal oxidase subunit 1
2T7 (sample 13)	3811554-3811555	3811363..3812229	CQW49_18005	hypothetical protein
2T8 (sample 12)	3942950-3942951	3942916..3944142	CQW49_18570	MFS transporter
2T9 (sample 18)	3973989-3973990	3973935..3974957	CQW49_18735	flagellar motor switch protein FliG
2T10 (sample 16)	4079252-409253	4078557..4079414	CQW49_19260	hypothetical protein
From phenotypic screening of second transposon library				
2T5 sample 20 (“white mutant 899”)	2150238-2150240	2149792..2151069	CQW49_10250	Methane monooxygenase/ammonia monooxygenase subunit

* Sample number as shown in Table 4.3.

4.3.4. Screening of the library for mutants altered in the reduction of selenite

The mutants of *Ms. trichosporium* OB3b were inoculated onto NMS agar with kanamycin (0.15 µg/ml final concentration) and 10 µg/mL of Se as Na_2 SeO_3 and grown in the presence of methane. The total mutant of colonies of the transposon library screened was 5,500. The colonies were growing well after 4 weeks, as shown in (Fig 4.8), there were colonies with different size and colour. The colonies with white colour may not have been able to reduce the selenite and there was a wide range of orange coloured colonies on the plates; these colonies were able to reduce selenite. White colonies (with numbers 839, 841, 885, 899, 900, and 901) as shown in Fig 4.8 were selected and tested again on NMS agar media and in liquid media, with 10 µg/mL Se as Na_2SeO_3.

When the white colonies were inoculated onto agar media, all the colonies in the plates were white in colour, but when a single colony from each plate was re-grown in NMS liquid media in flasks, all of them reduced selenite because the medium appearance changed to an orange colour, except one flask which was inoculated from colony number 899, which was white (indicated by the red box in Fig 4.8).

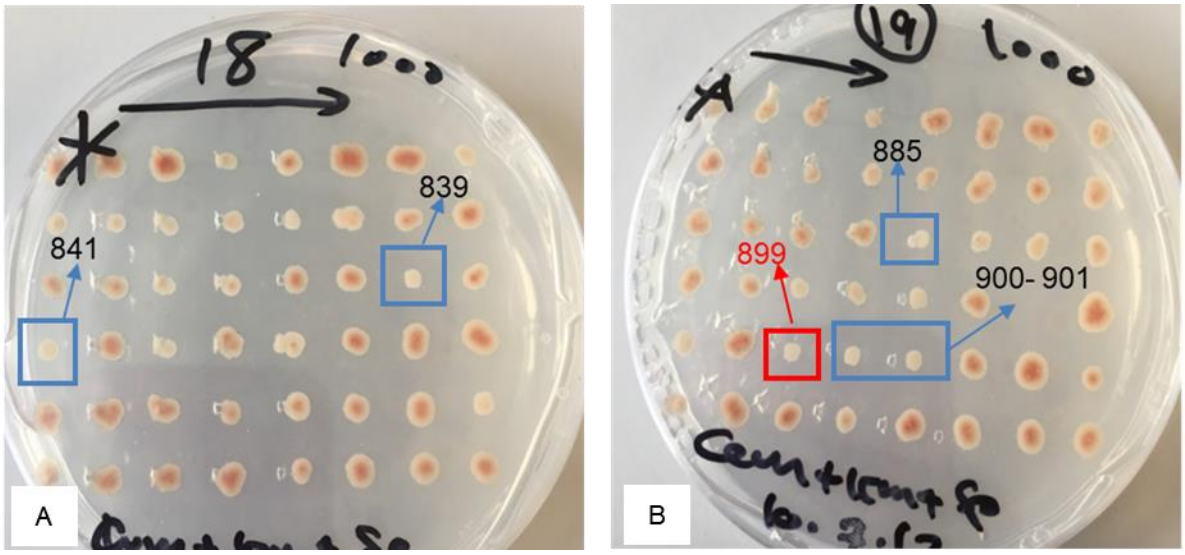


Figure 4-8. Growth of mutants of *Ms. trichosporium* OB3b

mutants grown on NMS agar containing Km and Se.15 µg/ml kanamycin and 10 µg/ml selenite. Mutants that could not reduce selenite appear as white colonies. The colonies which were the bacteria Pict for further analysis are shown with coloured boxes, the red box shows the mutant with subsequently confirmed as selenite reduction mins other mutants are shown in blue

.

To confirm that colony number 899 was unable to reduce the selenite, 1 ml of overnight cultured bacterial culture was inoculated into three flasks containing NMS medium with 10 µg/mL Se as Na_2SeO_3 in parallel with two controls (wild-type *Ms. trichosporium* OB3b, and another mutant from the transposon library that gave red colonies). This test confirmed that the colony number 899 cannot reduce the selenite in either agar and liquid media (Fig 4.9).

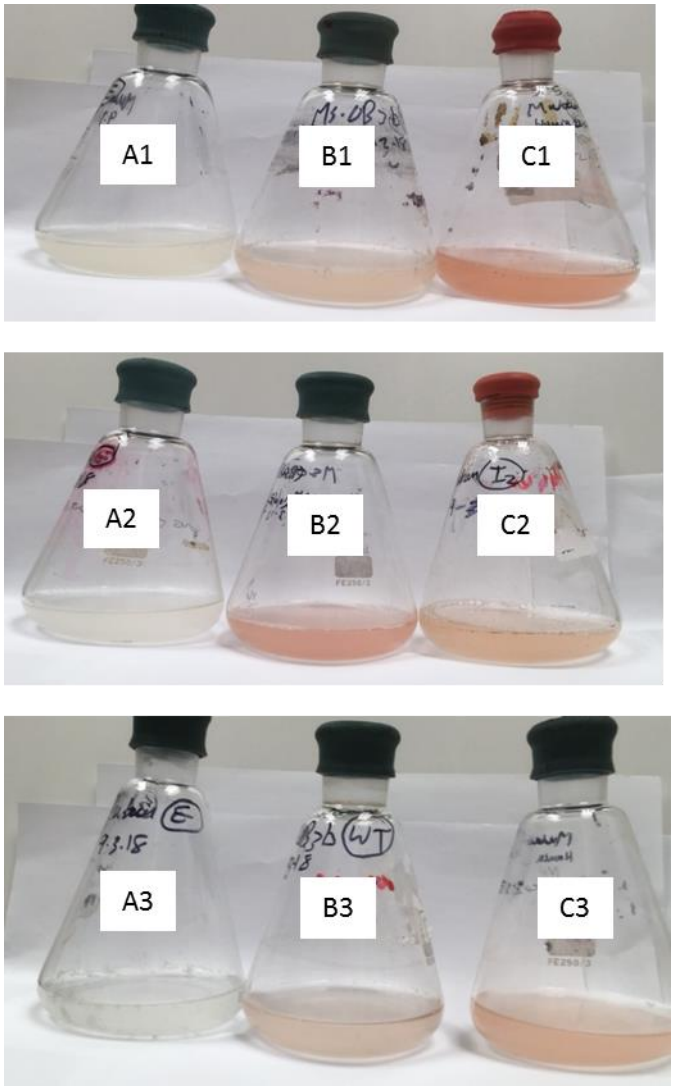


Figure 4-9. Selenite reduction

Triplicate experiment to show selenite reduction by the “white mutant” (colony number 899) (A1, A2, A3), wild-type *Ms. trichosporium* OB3b (B1, B2, B3) and the selenite reduction-positive (“red”) mutant (C1, C2, C3) in liquid media containing selenite.

4.3.5. Identification of a gene which is involved in reduction of selenite

The mutant which was unable to reduce the selenite in liquid media was sent for whole genome sequencing. The results showed that the position of the mariner transposon (Fig.4.5 and Table 4.4) this shown clone 2T5, which was derived from colony 899 was within the gene that spans from base 2149792

to 2151069 in the *Ms. trichosporium* OB3b reference genome. This gene encodes the subunit B of particulate methane monooxygenase.

4.3.6. Testing the role of the inactivated gene in selenite reduction

Since the chromosome of *Ms. trichosporium* OB3b has two copies of the pMMO operon (Gilbert *et al*., 2000), it was reasoned that the "white" transposon mutant 2T5 (which is inactivated in one of the two copies of *pmoB*) must still be able to grow using methane as growth substrate and may be impaired in the availabity of methane-derived electrons to reduce selenite. If this were correct, it may be possible to restore selenite reduction in the mutant by supplying formate, which the methanotrophs can use as an electron source via formate dehydrogenase.

When the "white" mutant (2T5, 899) was tested by growing with selenite and sodium formate, the result showed (Fig 4.10) that there was a substantial change in the colour of the culture to red when formate was added. Controls confirmed that there was no observable reaction between selenite and formate in the absence of methanotroph cells and that the wild-type strain reduced selenite to elemental selenium whether formate was present or not.

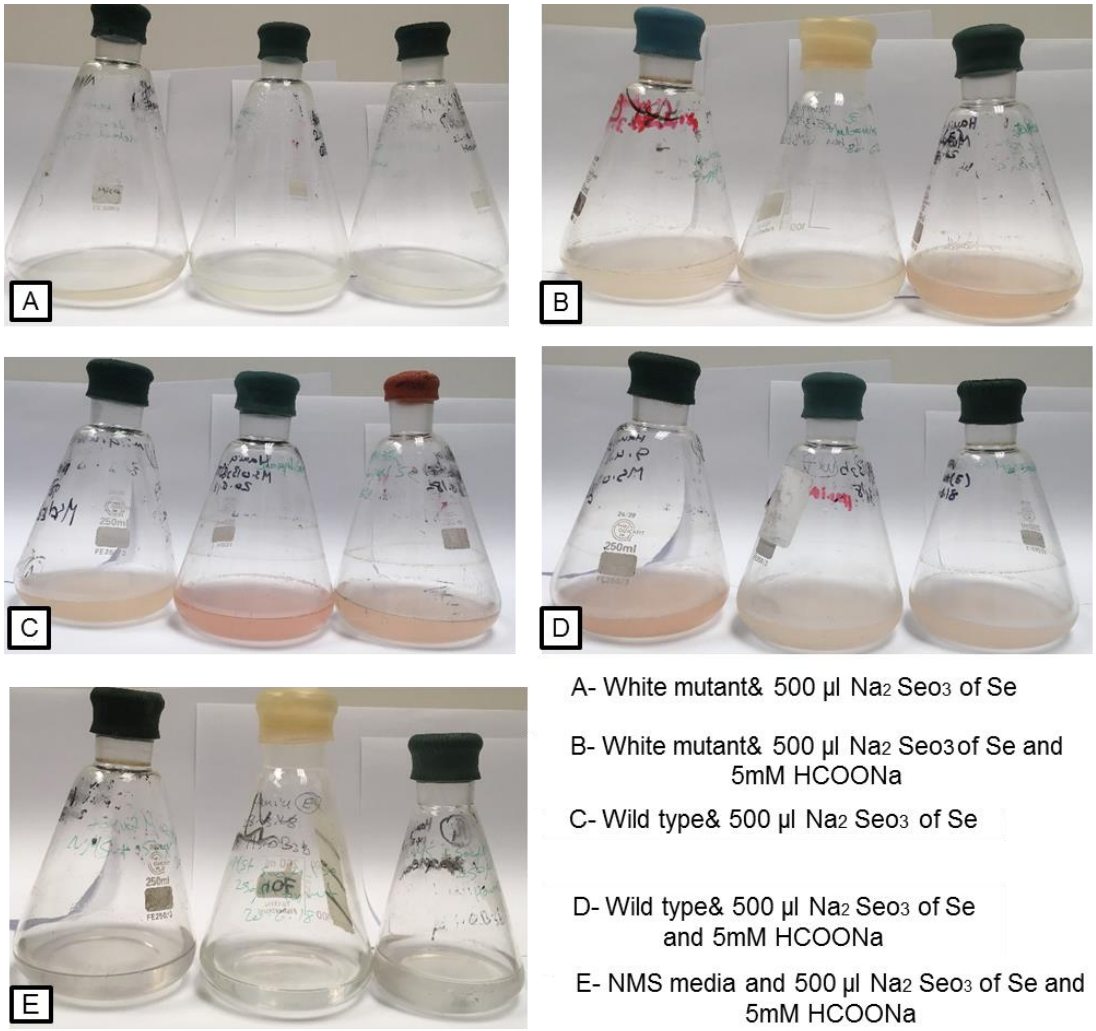


Figure 4-10. Testing the hypothesis that the selenite reduction in the white mutant can be restored by adding format.

4.4. Discussion

This study has identified a gene which is involved in the remediation of selenite $(SeO_3)^{2-}$ in the methanotroph *Ms. trichosporium* OB3b. Earlier research has shown that methanotrophic bacteria such as *Ms. trichosporium* OB3b and *Mc. capsulatus* (Bath) are able to reduce certain heavy elements. For example, *Methylococcus capsulatus* (Bath) reduces chromium VI (Al-Hasin *et al*. 2009), and both *Ms. trichosporium* OB3b and *Mc. capsulatus* (Bath) can reduce selenite $(SeO_3)^{2-}$ to components that are less toxic to the environment (Eswayah et *al*, 2016).

The PCR technique was used to with the intention of confirming whether the transposon had transferred from the plasmid to the chromosome of *Ms. trichosporium* OB3b. The result on the agarose gel showed there were no fragments of the transposon amplified by the primers for any of the chromosomal DNA samples, whilst the positive control with pSAM_R1 as the template gave the expected PCR product (Fig.4.1). Closer analysis of the sequence of pSAM_RI revealed that when the transoposon is transferred from the plasmid, part of the transposon sequence (including the gene for the enzyme transposase) is left behind. The primers that were used for PCR flanked the whole sequences of Transposon, including the transposase gene. This is why the primers did not recognize to the sequences of Transposon, and that is why no PCR product was observed on the gel from the putative transposon mutant chromosomal DNA preparations. However, this result was consistent with the transposon being transferred from the plasmid to the chromosome of *Methylosinus trichosporium* OB3b.

The Southern Blot Hybridization was selected to investigate the transposon insertion in the chromosome of mutants *Ms*. *trichosporium* OB3b. The DNA of *Ms. trichosporium* OB3b was purified twice via using two different kits to obtain a high concentration of DNA. The DNA of *Methylosinus trichosporium* OB3b was attempt to be digested by two types of restriction enzymes; however, the DNA did not cut (Fig 4.2 and 4.3). The reason for the difficulties in cutting the DNA of *Ms. trichosporium* OB3b by the restriction enzymes was not explored

in detail because it was decided to use the more powerful technique of whole genome sequencing to characterise the mutants.

Extraction and whole genome sequencing of DNA were used to confirm that the transposon had inserted in the *Methylosinus trichosporium* OB3b chromosome. 10 samples of the putative transposon in the mutant bacterial cells were sent to the Microbes NG team in the University of Birmingham together with a wild type *Methylosinus trichosporium* OB3b sample as a control. Genome sequence information was processed using Mauve contigs mover (MCM), the contig assemblies were based on comparison to the complete reference genome *Ms. trichosporium* OB3b Gen Bank accession. The result displayed an extra DNA sequence in the mutant draft genome.

The sequence of the transposon was BLAST-searched with each sequence of the putative transposon mutants. The BLASTn tool confirmed that the extra DNA is transposon with 99% of the similarity (Fig 4.4). Out of 8 mutants sequenced, only 3 insertion sites were observed. This result was surprising because it has been found in other organisms that the *Mariner* transposon insertion is highly random (Perry, Yost, 2014). Hence another 10 mutants from a new transposon mutant library were sequenced. Shorter conjugation times were explored to see whether the problem with multiple identical clones was that the recipient methanotrophs were multiplying during the 24-hour conjugation. It was found that shorter conjugation times were not effective, but when a new library produced after conjutating for 24 hours was analysed, the result showed, as intended, 10 different insertion sites in the *Ms. trichosporium* OB3b genome (Fig 4.6 and Table 4.4).

The red colour produced by elemental selenium was used to screen for mutants in genes involved in reducing selenite. When many of the mutants from the transposon library that appeared white on selenite-containing agar were re-grown on agar media containing kanamycin and selenite, most of colonies were red as observed for the wild-type strain by Eswayah *et al*. (2017). However, some white colonies retained the white phenotype on re-

culturing on the same NMS agar (supplied with 15µg/ml kanamycin and 10 µg/mL as Se as Na_2SeO_3; Fig 4.7).

To confirm whether the white colonies were indeed unable to reduce selenite, they were inoculated into both NMS agar and liquid NMS media (both provided with kanamycin and selenite). These white mutants remained white when re-grown on solid medium but all, with the exception of colony number 899 (2T5, Fig 4.7) were red in colour when grown in liquid medium.

The colony 899 (clone 2T5) was therefore chosen for further analysis. It was shown that this mutant was unable to reduce the selenite even in the absence of kanamycin. One explanation for the colonies which were able to reduce the selenite in the agar media and could not reduce it in liquid media may be because they grow differently under the different conditions. This may be a similar phenomenon to that observed by Knapp *et al*., (2017) these workers examined 90 garden soils from Western Australia to evaluate predictions of antibiotic resistance genes and relationships between metals and genes by comparing the concentrations of 12 metals and 13 genes related to tetracycline, beta-lactam and sulphonamide resistance. Here, different results were found depending on whether solid or liquid media were used to test the resistance of the isolates.

Colony number 899 was inoculated again in the liquid media with appropriate controls. The flasks contained the white colony compared with control (WT) were not changed colour (Fig 4.8). The genomic DNA of the “white mutant 899” was sequenced and the result was analysed via programs; Mauve and Blast.

It was surprising that the same gene (*pmoB*) which is involved in reduction of selenite is itself involved in methane oxidation (Gilbert *et al*, 2000). This led to a hypothesis that the lack of selenite reduction in this mutant, presumably with reduced methane monooxygenase activity, was due to lower availability of electrons from methane available to reduce selenite, compared to the wild-type. Sodium formate (an electron donor that methanotrophs can use) was

selected as alternative electron donor and added into the NMS liquid media with selenite and incubated with presence of methane. The colours of the cultures indicated that selenite reduction in the mutant could be restored by adding formate (Fig 4.9 B). This may mean that bacterial cells of mutant 899 (clone 2T5) may be able to recover the selenite reducing function by obtaining electrons from sodium formate rather than methane. A negative control confirmed that there was no reaction between the sodium formate and sodium selenite in the absence of bacteria, even at 30 °C for one month (Fig 4.9 B).

Hence, a major achievement of this study is that for the first time, it has been shown that the methanotraphs with a critical role in the global methane cycle can be studied via transposon mutagenesis and also that the ability to reduce selenite to elemental selenium can be abolished by inactivation of one of the copies of *pmoB*.

The transposon mutagenesis work described in this and the preceding chapter did not identify a gene that appeared to be solely involved in selenite reduction. In order to determine whether a protein involved in selenite reduction could be identified it was decided to study the selenite reduction reaction biochemically in greater detail, as described in the following chapter.

Chapter 5 The physiology of the selenite reduction reaction

5.1. Introduction

Pollutants in the environment are a major human health concern. Selenium (Se) is an essential element which is needed in limited quantities for normal health and function, Se is toxic at high concentrations for most living organisms. Selenium components that filter from rocks are significantly pollutants in the environment. Kessi and Hanselmann (2006) showed that microorganisms can carry out the conversion of SeO_3^{2-} to Se^0 through different mechanisms. Se is subject to various microbial transformations, some of which are important in terms of bioremediation.

Microorganisms play an essential role in the selenium cycle in the environment. It is well known that selenium (Se) is an essential trace element for microorganisms and multicellular organisms, though it is toxic at high concentrations. *Streptomyces* sp. ES was isolated from a selenium mining soil in South West China (Tan *et al*., 2016). This strain, which produced elemental selenium nanoparticles from selenite, was identified as *Streptomyces sp.* based on 16S rRNA gene sequence, physiological and morphological characteristics. Such microorganisms might be explored as potential biofactories for synthesis of metal (loid) nanoparticles. (Tugarova *et al*., 2013) also reported a strain of *Azospirillum brasilense* able to reduce selenite to elemental selenium. Subsequently, the same group reported that cell lysis was the origin of the extracellular Se^0 particles, which initially formed within the cells of this strain (Tugarova *et al.,* 2014).

The production of the red colour from selenite was detected in the cell wall fraction of *Ms. trichosporium* OB3b, and a weak red tint in the cell membrane fraction which was attributed to traces of reductase enzyme(s) contamination which may have spread from the cell wall to the cell membrane (Dhanjal & Cameotra 2010, Eswayah *et al.*, 2017).

In Chapter 4, it was shown that a mutant that is inactivated in one of the copies of the major subunit of pMMO had diminished selenite reduction activity. This was consistent with the observation that methane is needed for the reduction of selenite, though it did not show how electrons pass from the methane

oxidation pathway into the reduction of selenite. In order better to understand this process, the location of the selenite-reducing activity within the cell was investigated again, before further investigation of the nature of the selenite-reducing species.

5.2. Material and Method

5.2.1. Cell fractionation of *Ms. trichosporium* OB3b

To determine the position of selenite reduction in the *Ms. trichosporium* OB3b cells, the grown culture (OD_{600nm}~ 0.7) of *Ms. trichosporium* OB3b was centrifuged at 11,000 x *g* for 10 min at 4ºC to obtain a pellet. The pellet was washed with ice-cold 50 mM Tris-HCl (pH 7.5) and re-suspended in 10 mL of the same buffer and protease inhibitor cocktail (1% v/v) (Sigma-Aldrich, Dorset, UK) was added. The suspension was passed through a French Pressure cell (8.2 MPa, 4ºC). The broken cell suspension was then fractionated by a modification of the method reported by Smith and Foster (1995), as follows: the whole procedure was performed at 0 to 4ºC to minimize protein degradation. The broken cell suspension was centrifuged (3,000 x *g*, twice for 2 min each) to remove debris before being centrifuged (27,000 x *g*, 20 min) to sediment cell wall fragments. The cell walls were washed by resuspension in 50 mM Tris-HCl (pH 7.5) and kept in the same buffer. The supernatant fraction was centrifuged again (27,000 x *g*, 20 min) to remove remaining cell wall material. Membrane fragments were sedimented by centrifugation (105,000 x *g*, 60 min) of the supernatant. The pellet (cell membranes) was washed in 50 mM Tris HCl (pH 7.5), centrifuged again under the same conditions, and re-suspended in the same buffer. The supernatant from the first ultracentrifugation was centrifuged again under the same conditions to remove remaining membranous material and kept as the cytoplasmic fraction.

5.2.1.1 Measurement the concentration of protein in the cells by the BCA assay

The BCA assay kit was used to measure the total of protein in each fraction of *Ms. trichosporium* OB3b, which were cell wall, cell membrane and cytoplasm. The BSA standards were prepared from a stock solution of 0.01 g/ml bovine serum albumin in deionised water. Six Eppendorf tube were labelled and then quantities of BSA stock solution were added into deionised water as indicated

in Table 5.1. The BCA reagent was prepared by adding 8 ml of bicinchoninic acid solution to 0.16 ml of copper sulphate (supplied by Sgma) and mixed. After that, 25 µl of each sample were mixed with 200 µl of BCA reagent in the wells of a 96-well plate, and then the plate was incubated at 37°C for 30 minutes. The absorbance of each sample was measured at 570 nm. The absorbance readings were plotted against the concentration BSA to prepare a standard curve.

Table 5-1. Quantities of BSA and water with protein standard curve.

Deionised H_2O	990 µl	980 µl	960 µl	940 µl	920 µl	900 µl
BSA (10 mg/ml)	10µl	20 µl	40 µl	60 µl	80 µl	100 µl
Concentration (mg/ml)	0.1	0.2	0.4	0.6	0.8	1

5.2.1.2. Determining the location of the selenite reduction activity in *Ms. trichosporium* OB3b

Two different approaches were used to determine the distribution of selenite reduction activity within the cellular fractions (cell wall, cell membrane and cytoplasm) of *Ms. trichosporium* OB3b. Initially, following the method of Eswayah *et al.* (2017), the same volume of each fraction (200 µl) was used and mixed separately with 30 µl of Na $(SeO_3)_2$ solution (Se concentration 1000 mg/L) and 70 µl (50 mM)Tris-HCl buffer pH 7.5, then the suspension for each fraction was transferred to 96-well plate in the presence of appropriate negative controls (each reaction with 30 µl of the same buffer replacing the selenite solution), and directly the plate was incubated at 30 °C for four days.

In the second, the amount of each fraction was adjusted based on the volumes of each fraction produced during the fractionation procedure, so that each corresponded to the same proportion of the original culture. The total volumes of the fractions were 1, 5 and 25 ml for the cell wall, cell membrane and cytoplasm, respectively. Hence, in the 96-well plate assay the volumes of the

fraction used were fixed in the same propoprtion at 8 µl for the cell wall fraction, 40 µl for the cell membrane fraction, and 200 µl for the cytoplasm fraction. Each of these samples was made up to 200 µl with 50 mM Tris-HCl buffer (pH 7.5) and then mixed with 30 µl Na $(SeO_3)_2$ (Se concentration 1000 mg/L) to give a total reaction volume of 300 µl respectively and negative controls were set up with the same volumes of each fraction in the same total reaction volume, without addition of selenite. The 96-well plate was incubated at 30°C for one week.

5.2.2. Investigating whether the selenite-reducing molecule(s) in *Ms. trichosporium* are sensitive to protease digestion

To test that if an enzyme (i.e. a protein) is responsible for reducing selenite in the *Ms. trichosporium* cells, the protease trypsin was selected to digest the proteins in the cell fraction. A trypsin solution (1 mg/ml) was prepared in 1 mM HCl. Four experimental samples were prepared in a 96-well plate (Table 2). All the components of the reaction except the Na_2 $(SeO_3)_2$ were mixed for each sample and then the reactions were incubated overnight at 30 ºC. Before adding the Na_2 $(SeO_3)_2$, 20 µl of the reaction were stored in the freezer at -80 ºC from Sample B (which has cytoplasm fraction and trypsin; to analyse it later as a control when the samples were run on the SDS gel) and then after addition of the Na_2 $(SeO_3)_2$, the 96-well plates were incubated at 30 ºC for 5 days. The experiment was subsequently repeated with an increased amount of trypsin (7.65 µl in replace of 5.65 µl in Table 5.2), together with an additional control to investigate the role of endogenous proteases from the cytoplasm in digesting the proteins.

Table 5-2. Quantities of materials to investigate whether a protein is required for the selenite-reducing activity

Sample	**Quantities of material**
Sample A	200 µl cytoplasm fraction, 30 µl $Na_2 (SeO_3)_2$ (1000 mg/L with respect to Se), 70 µl (50 mM Tris-HCl, pH 7.5) buffer
Sample B	200 µl cytoplasm fraction, 30 µl $Na_2 (SeO_3)_2$ (1000 mg/L with respect to Se), 5.65 µl trypsin, 64.35 µl buffer (50 mM Tris-HCl, pH 7.5).
Sample C	264.35 µl Buffer (50 mM Tris-HCl, pH 7.5), 5.65 µl trypsin, 30 µl $Na_2 (SeO_3)_2$ (1000 mg/L with respect to Se).
Sample D	294.35 µl Buffer (50 mM Tris-HCl, pH 7.5), 5.65 µl trypsin

5.2.3. SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide gel Electrophoresis)

SDS-PAGE was used to test whether trypsin had digested the proteins in the previous experiment. 20 µl from reaction B (the sample from the previous experiment that had been stored in the freezer before adding the selenite) and the same amount from the original sample of cytoplasm fraction without addition of trypsin (as a negative) control were mixed with 7 µl of loading dye, then incubated at 95 ºC to for 10 minutes (to denaturature the proteins). The samples (27 µl) were applied into the wells of the SDS-PAGE and the gel (Bio Rad) was run at 100 V with buffer containing 0.02% Nan_3 for one hour. To display clear bands of the obtained protein on the gel the, Silver Stain Kit (Thermo Scientific) was used, as follows.

The gel was washed twice in ultrapure water for 5 minutes each time and then fixed in a solution of 30% (v/v) ethanol and 10% (v/v) acetic acid in water, twice for 15 minutes. The gel was washed with 10% (v/v) ethanol solution for 5

minutes, after that washed by ultrapure water twice. The Sensitizer was prepared to make the working solution by mixing 50 µl Sensitizer with 25 ml ultrapure water, then the gel was incubated in the Sensitizer working solution for 1 minute, then the gel was washed with two changes of ultrapure water for 1 minute each. Stain working solution was prepared by mixing 500 µl of Silver stain solution with 25 ml of ultrapure water prepare, after that the gel was incubated in the working stain solution for 30 minutes. The gel was washed rapidly with ultrapure water for 20 seconds two times and immediately the gel was incubated in Developer solution until protein bands appeared. To stop the development of the gel, the developer was replaced with a 5 % (v/v) solution of acetic acid for 10 minutes. The plate containing the gel was then stored at 4 ºC in the fridge in ultrapure water.

5.3. Results

5.3.1. Cell fractionation of *Ms. trichosporium* OB3b

Cells of *Ms. trichosporium* OB3b were fractioned from 50 ml of culture as detailed above. This yielded 1 ml of cell wall fraction, 25 ml of cytoplasm fraction and 5 ml of the cell membrane fraction, each in 50 mM Tris-HCl (pH 7.5).

5.3.1.1. Concentration of protein for each fraction by BCA assay

To determine the concentration of protein in each cellular fraction of *Ms. trichosporium* OB3b, the BCA assay was used with BSA standards, as shown in Table 5.3.

Table 5-3. BSA standards from 0 to 1 mg/ml (reading by spectrometer) in the 96-well plate

Concentration Of BSA	**Reading of spectrometer**	**Concentration Of BSA**	**Reading of spectrometer**
0.1 mg/ml	0.258	0.6 mg/ml	0.799
	0.254		0.830
	0.270		0.848
0.2 mg/ml	0.377	0.8 mg/ml	0.967
	0.391		1.056
	0.383		1.005
0.4 mg/ml	0.631	1 mg/ml	1.213
	0.631		1.311
	0.664		1.206

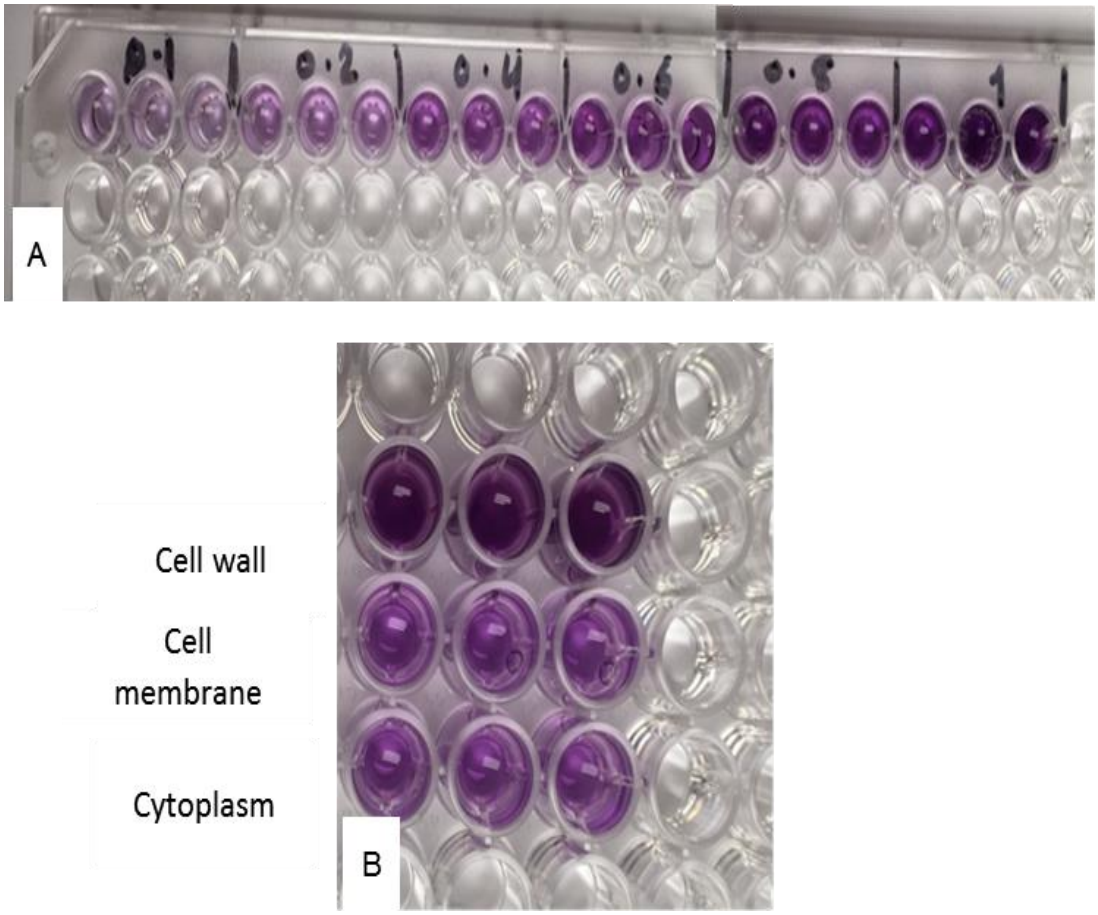


Figure 5-1. 96-well plates used to measure the protein condensations.

96-well plate used to measure the BSA standards (A) and each fraction of *Ms. trichosporium* OB3b (B)

Table 5-4. The concentration of protein in the fractions of *Ms. trichosporium* OB3b in the 96-well plate from figure 1B

Fractions of *Ms. trichosporium* OB3b	**Concentration of protein in well 1**	**Concentration of protein in well 2**	**Concentration of protein in well 3**
Cell wall	1.219 mg/ml	1.222 mg/ml	1.230 mg/ml
Cell membrane	0.609 mg/ml	0.581 mg/ml	0.577 mg/ml
Cytoplasm	0.564 mg/ml	0.570 mg/ml	0.562 mg/ml

Table 5-5. The concentration of protein in original volume for each fraction.

Fraction	**Original volume**	**Average of Concentration of protein**
Cell wall	1 ml	1.22 mg/ml
Cell membrane	5 ml	0.589 mg/ml
Cytoplasm	25 ml	0.565 mg/ml

By comparison between BSA standards (Table 5.3) and absorbance of the assays of the fractions of *Ms. trichosporium* OB3b (Fig 5.1) (Table 5.5), the concentration of the protein in the cell wall fraction was the highest 1.22 $\pm$ 0.006 mg/ml, then the cell membrane was 0.589 $\pm$ 0.017 mg/ml and the concentration of cytoplasm was 0.565 $\pm$ 0.004 mg/ml.

5.3.1.2. Location of selenite reduction activity in the cells of *Ms.trichosporium* OB3b

To determine the location of the selenite reducing activity, the cells of *Ms. trichosporium* OB3b were fractioned into three parts which are cell wall, cell membrane and cytoplasm, as detailed above.

When an equal volume of each fraction was tested by adding 30 µl $Na_2(SeO_3)_2$ (1000 mg/L with respect to Se), and incubating in the 96-well plate at 30 ºC, the fraction of cell wall appeared orange in contrast to the control (each fraction just with buffer) after three days as shown in (Fig 5.2 A), but orange colour appeared after six days in the cell membrane and cytoplasm as shown in (Fig 5.2 B). This result showed there is some activity reducing the selenite in all of the fractions of *Ms. trichosporium* OB3b cells.

When the volumes of each fraction were adjusted so that the amount of each fraction corresponded to the material from the same volume of original culture (Fig 5.3) the selenite reduction activity was observed within a period of 4 days in the cytoplasm alone. Hence, whilst there is clearly detectable selenite reduction activity in all fractions, the amount of activity in the cells is predominantly in the cytoplasm.

In an attempt to quantify the selenium produced by the reduction reaction, UV-visible spectra for each of the wells in (Fig 5.3) were recorded using the microplate reader (Fig 5.4). The spectra of the cytoplasm samples that visibly showed the production of red elemental selenium were not distinguishable from the parallel control with no added selenite. Hence, such spectrophotometric analysis cannot be used to quantify the production of red elemental selenium in these reactions.

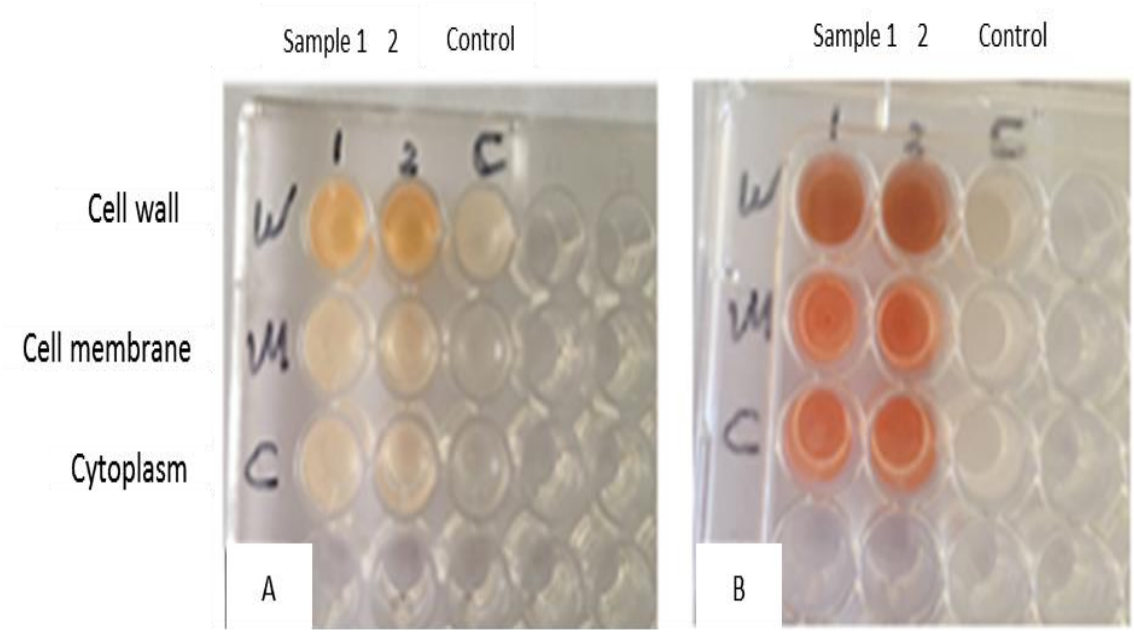


Figure 5-2. The reduction of selenite in the fractions of *Ms. trichosporium* OB3b.

After three days of incubation, the reduction was clear in the bacterial cell wall (A), after 6 days the reduction was observed in the all fractions(B). Samples with selenite (A and B), negative control C buffer without selenite

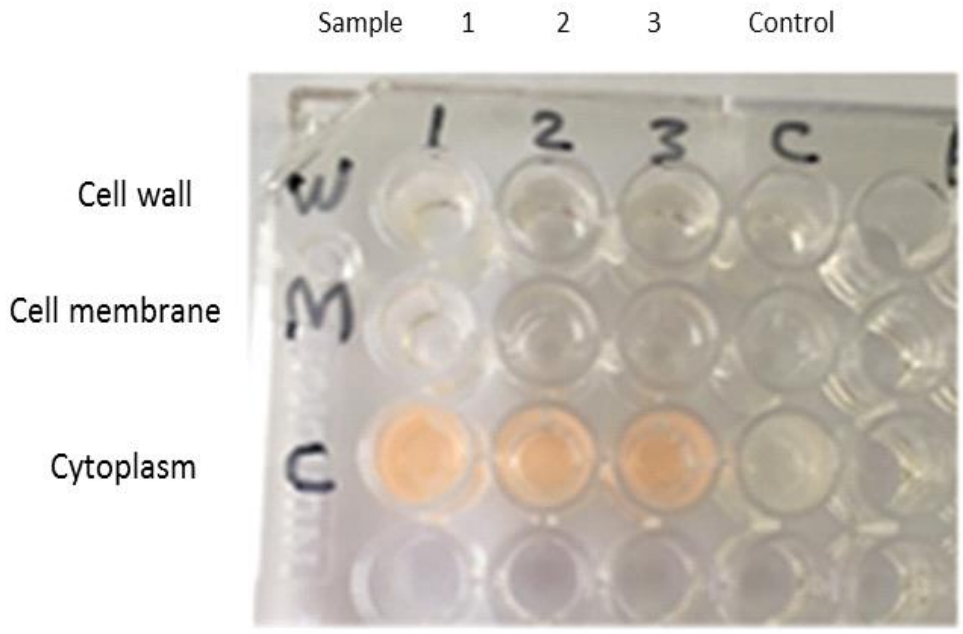


Figure 5-3. The activity of reduction selenite was primarily in the cytoplasm after 4 days of incubation

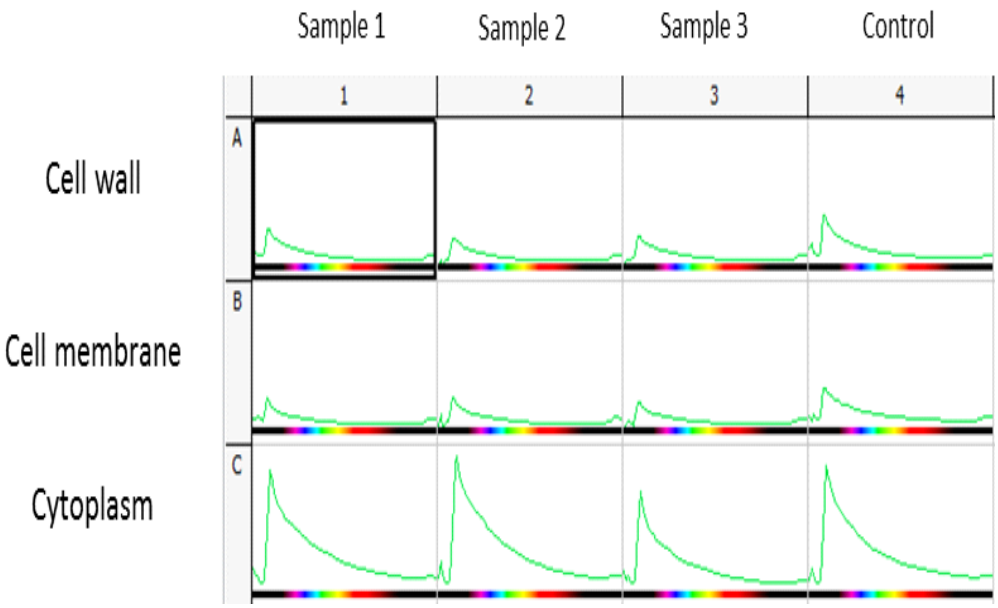


Figure 5-4. Spectrophotometric scan of reduction reactions

Spectrophotometric scan of reduction reaction. spectrophotometric scans of the plate shown in Fig 5-3. The vertical axes shown absorption and the horizontal axes shows the waving of light

5.3.2. Investigation of the molecule involved in reducing selenite

The results above showed that the cytoplasm was reducing the selenite in the cells of *Ms. trichosporium* OB3b. The next step was to investigate which type of molecule was involved in reducing the selenite. Therefore, the effect of a protease (trypsin) was investigated to determine whether one or more enzymes might mediate the reduction of selenite. Following treatment with trypsin, the cytoplasm fraction still reduced the selenite. A control reaction confirmed that the trypsin solution alone did not reduce selenite (Fig 5.5). SDS-PAGE analysis showed that the proteins in the trypsin-treated samples had undergone substantial degradation compared with the untreated cytoplasm fraction (Fig 5.6). This suggested that the molecule(s) responsible for selenite

reduction were either not proteins or were in the small amount of protein that remained after digestion.

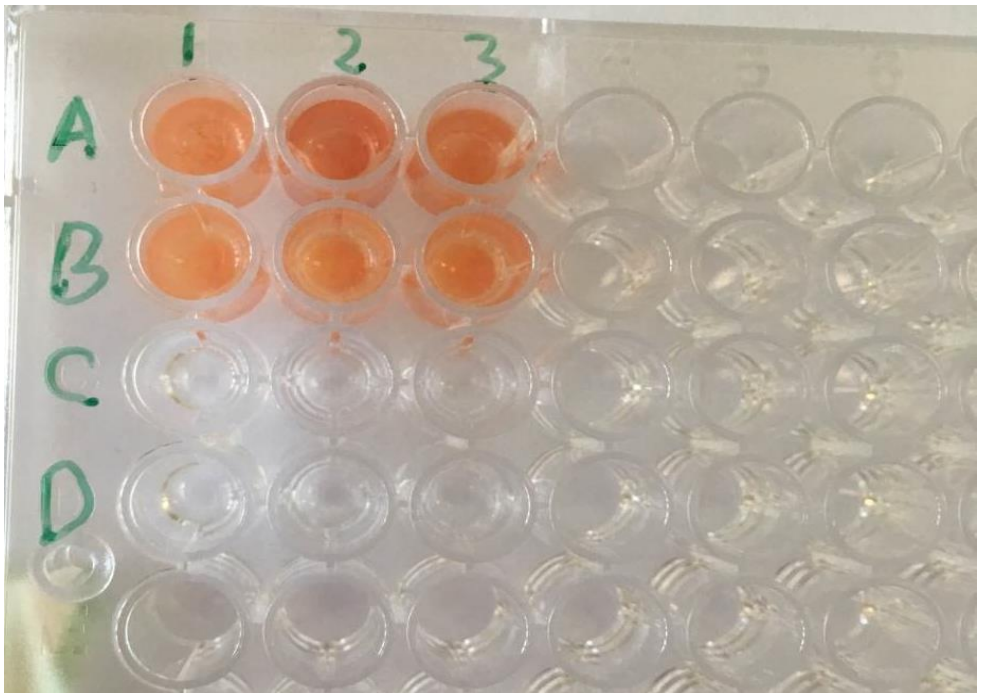


Figure 5-5. Investigating the effect of trypsin on the selenite-reducing activity of the cytoplasm fraction.

Cytoplasm with selenite (A), cytoplasm with selenite and trypsin (B), buffer and trypsin with selenite (C), buffer and trypsin (D). Each trypsin-containing reaction contained 5.65 µl of trypsin solution, as detailed in the Methods section. Each experiment had 3 replicates, as shown in the figure.

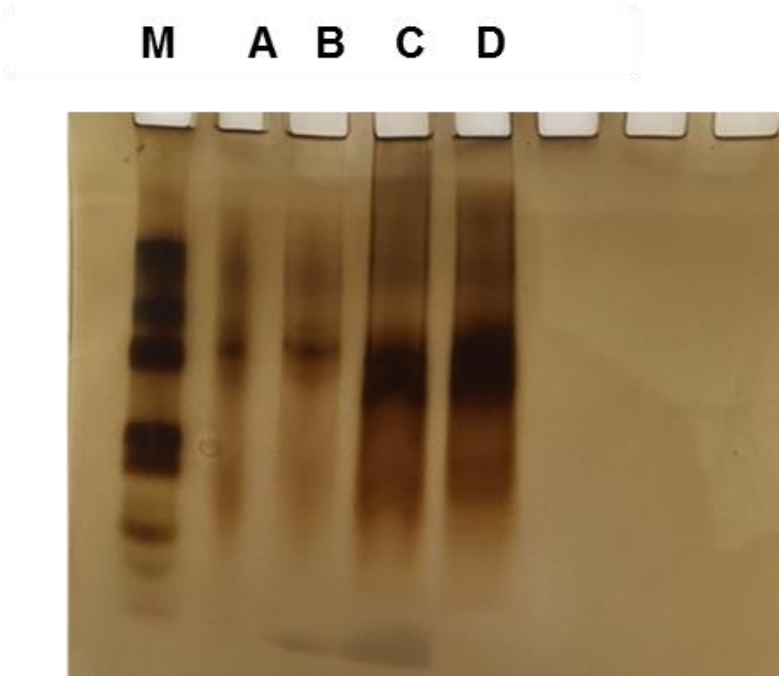


Figure 5-6. SDS-PAGE showing digestion of proteins with 5.65 µl of trypsin.

SDS-PAGE showing molecular marker (M), samples of cytoplasm incubated overnight with trypsin (A & B), untreated cytoplasm (C & D) control

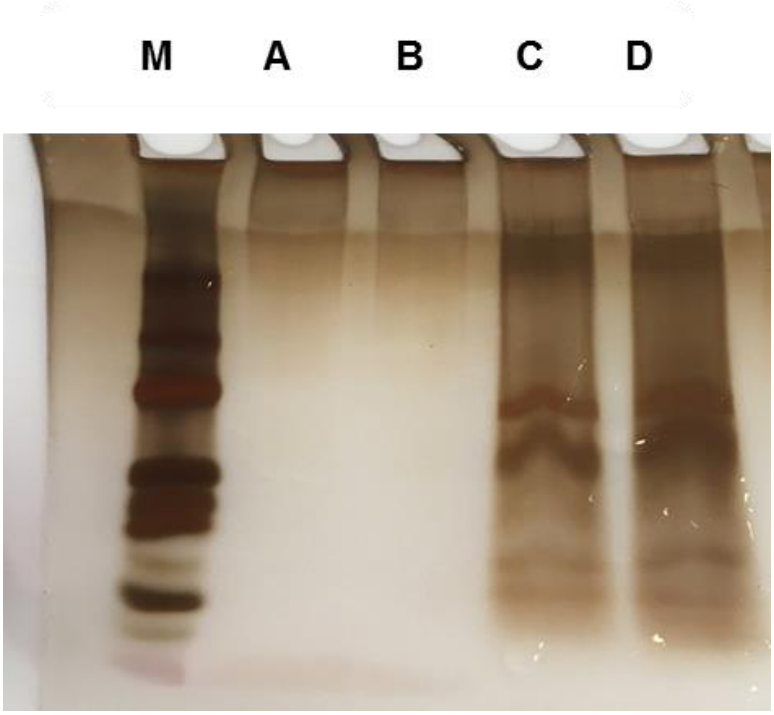


Figure 5-7. SDS-PAGE showing digestion of proteins with 7.65 µl of trypsin.

Marker (M), cytoplasm and buffer incubated overnight without trypsin (A), cytoplasm and buffer incubated overnight with trypsin (B), and untreated cytoplasm (C & D).

Another experiment was performed to determine whether the digestion of proteins in the experiment was due to the added trypsin. Also, the volume of trypsin solution was increased to 7.65 µl in order to obtain more nearly complete digestion. Here, the control cytoplasm fraction sample was incubated under the same conditions as the experimental sample, except that no trypsin was added. Under these conditions, digestion of the protein in the fraction was seen even when trypsin was not added (Fig 5.7). This indicates that there are proteases in the cytoplasm fraction that are able to digest essentially all of the protein in the fraction. Nonetheless, the result is consistent with the molecule (s) directly responsible for reduction of selenite being non-protein moieties.

5.4. Discussion

The major focus of this chapter was to analyse the cell fractions of wild type (cell wall, cell membrane and cytoplasm) of *Ms. trichosporium* OB3b and their interaction with selenite. The concentration of protein was measured for each fraction, because from previous studies it was possible that a protein was involved in reducing selenite (Eswayah *et al.,* 2017). Fractions were tested separately with selenite and monitored visually so, when the same volume of each fractions was exposed to selenite, the reducing activity of selenite was found to be primarily in the cell wall. Then after a longer incubation period, the red colour was observed in all the fractions.

The previous study showed that the selenite reduction activity was greatest in the cell wall fraction (Eswayah *et al.,* 2017). Transmission electron microscopy (TEM) analysis showed that the nanoparticles are found mainly on the surface of the cells. This suggested that selenite reduction may occur extracellularly or within the cell wall. Here, by compensating for the difference in the volumes of the different fractions, it has been shown that within the cell, whist all compartments appear to contain measurable selenite reduction activity, the greatest amount of activity lies in the cytoplasm.

The hypothesis that a protein is responsible for reducing the selenite in the cytoplasm was tested. The protein in the cytoplasm was digested by trypsin with overnight incubation but the selenite reducing activity remained In fact, the SDS-PAGE showed that the protein was digested almost completely even if trypsin was not added. These results indicate that the activity that reduces selenite in the cytoplasm fraction of cells of *Ms. trichosporium* OB3b does not depend on the immediate presence of any protein that can be digest by a protease.

The results presented in this study suggest that this work should be repeated to tested the genome for the presence of trypsin-resistant proteins and also test the dps genes/proteins (DNA protein) present in the genome or not.

Several studies have investigated the mechanisms of microbial formation of Se nanoparticles, where it has been found that Se nanoparticles are associated with proteins (Jain et al., 2015b; Song et al., 2017; Li et al., 2014).

However, Eswayah *et al.,* 2017 suggested that the protein responsible to reduce the selenite and they used a heat treatment to digested any protein in the cell wall fraction.

Chapter 6 General discussion and future directions

6.1 General discussion

The previous studies have shown that methanotrophs are able to reduce the activity of selenite to the less toxic elemental selenium in the presence of methane as a sole carbon and energy source. The overall aim of the studies reported in this thesis was for the first time to use transposon mutagenesis to identify genes that are responsible for the remediation of selenite $(SeO_3)^{2-}$ in methanotrophs and develop a system for transposon mutagenesis of methanotrophs by using the *mariner* transposon in pSAM_R1 to create a library of mutants of *Ms trichosporium* OB3b. To complement this research the physiology of selenite reduction was also investigated.

It was also intended to use the transposon mutagenesis system to investigate the genes needed for reduction of chromium (VI) to chromium (III) in methanotrophs. However, it was found (Chapter 2) that the colorimetric assay for chromium (VI) was less suited to development as a high-throughput screening system that the selenite reduction reaction, which produces a red colour without work-up. Also, the *Ms. trichosporium* strain, which was found to be more amenable to genetic manipulation, does not show the chromium (VI) reduction activity in whole cells. Nonetheless, the results reported in Chapter 2 confirmed that *Mc. capsulatus* (Bath) is able to reduce chromium (VI) to less toxic chromium (III) as seen in Fig. 2.2. This is consistent with the previous study (Al-Hasin *et al.,* 2009).

Results reported in Chapter 2 have confirmed that the methanotrophs *Ms. trichosporium* OB3b is able to reduce selenite form high toxic to less toxic (elemental selenium) form. This was observed as a change in the colour of liquid media to red as shown in Fig 2.3 (A and B). Also, the colonies on the plates became red in colour in the presence of selenite (Fig 2.3, C and D) as observed previously, Se reduction shown to produce extracellular Se reduced as shown in chapter 1 Fig 1.2 by TEM (Eswayah *et al.*, 2017).

The major achievement of chapter three and four, in this study for the first time, has been to show that a transposon library can be constructed in a methanotrophs, which is a representative of the wide diversity of environmental methanotrophs with a critical role in the global methane cycle.

Studies in organisms with well-developed genetic systems have recently been able to screen very large number of transposon insertion clones (more than 100,000 in some instances) to obtain high coverage of the genome (Perry and Yost, 2014; Van Opijnen and Camilli, 2013). Other studies have found that smaller libraries, of as few as 2,000 clones, can be used to obtain mutants impaired in specific processes where genetic systems are less well developed or screening procedures are difficult, for example requiring individual experimental animals such as in the study reported by Autret *et al.* (2001). In another example, Bharani *et al* (2014) used a transposon mutatgeneis methodology to transfer transposon IS-O-Km/hah from E*. coli S*17 to *Pseudomonas* strains *Pph* 1302A and 1448A and to screen the progeny for their plant colonization ability. By analysis of only 1,920 mutants, they were able to show that the disruption of *pyrB* by the transposon severely reduced the ability of Pph to grow in association with the plant.

Here, the target was to construct and screen around 10,000 mutants in the methanotroph system, which would have given approximately 87.9 % coverage of genes in the genome. The number of mutants actually screened was 5,500, which is estimated to have given approximately 43.5 % coverage of genes in the chromosome. Although this was fewer than intended, it was sufficient to enable isolation of a mutant impaired in selenite reduction.

The red colour produced by elemental selenium was used to screen for mutants in genes involved in reducing selenite. The results of the mutants from the transposon library appeared some white colonies on selenite-containing agar, although most of colonies were red as observed for the wild-type strain by Eswayah *et al*. (2017).

The colony 899 (clone 2T5) was one of a substantial number of colonies that appeared white on the initial screen. It was not completely clear at this stage that it had a substantially altered phenotype, due in part to the fact that smaller

colonies were generally less red in colour than large ones. Further analysis in liquid media with appropriate controls confirmed that the mutant 2T5 was impaired in reduction of selenite (Chapter 4). It was surprising that the gene interrupted by the transposon in this mutant was one of the copies of *pmoB*, encoding the large subunit of pMMO (Gilbert *et al*, 2000) (Fig 6.1). The insertion of the transposon into the *pmoB* gene interrupts the 1,278-bp gene at the 448th base pair. This will interrupt the PmoB protein at amino acid 149 (out of a total of 425 amino acids in the full protein). This would remove the transmembrane portions of PmoB but would retain the cytoplasmic domain of the PmoB, which is believed to contain the active site for oxidation of methane and other substrates (Balasubramanian et al., 2010). The first 425 amino acids of PmoB contain the residues implicated in binding the copper centres in the enzyme. Hence, it is predicted to abolish the association of PmoB with the membrane and likely the rest of the pMMO protein, but it is conceivable that the fragment of PmoB that could be expressed from the interrupted gene may have monooxygenase or other enzyme activity. The mutant is presumably able to assemble fully functional pMMO because the strain retains the other copy of the pMMO operon (Matsen *et al*., 2013; Gilbert *et al*., 2000).

Hence, it may be that the white mutant's phenotype is not due to inactivation of an enzyme which is specifically involved in reducing selenite to elemental selenium, but due to a more general lesion in the metabolism of the methanotroph. This leads to the question: is the reduction of selenite directly by MMO or indirectly? There are two possible explanations. One of them is that the sMMO is not the enzyme that directly the reduces the selenite, and so when the electrons are fed via another route from formate via formate dehydrogenase, then this provides electrons that can reduce the selenite by whatever enzyme or other system it is that performs the reaction. The other possibility is that the MMO does reduce the selenite directly but there is completely different mechanism by which selenite can be reduced which is only operational when format is added into the culture and that is independent of MMO. An extra piece of evidence in support of the first hypothesis is the observation that there is selenite-reducing activity in the cytoplasm, whereas pMMO resides in the membrane (Fig 6.1). The hypothesis here is the reduction

of selenite in the methanotrophs directly due to the MMO or not? The results presented in Chapter 4 (Fig 4.10 B) showed that the reduction of selenite is not directly due to the enzyme MMO because it can be restored by adding an alternative reducing agent (formate) even in the absence of the copy of MMO.

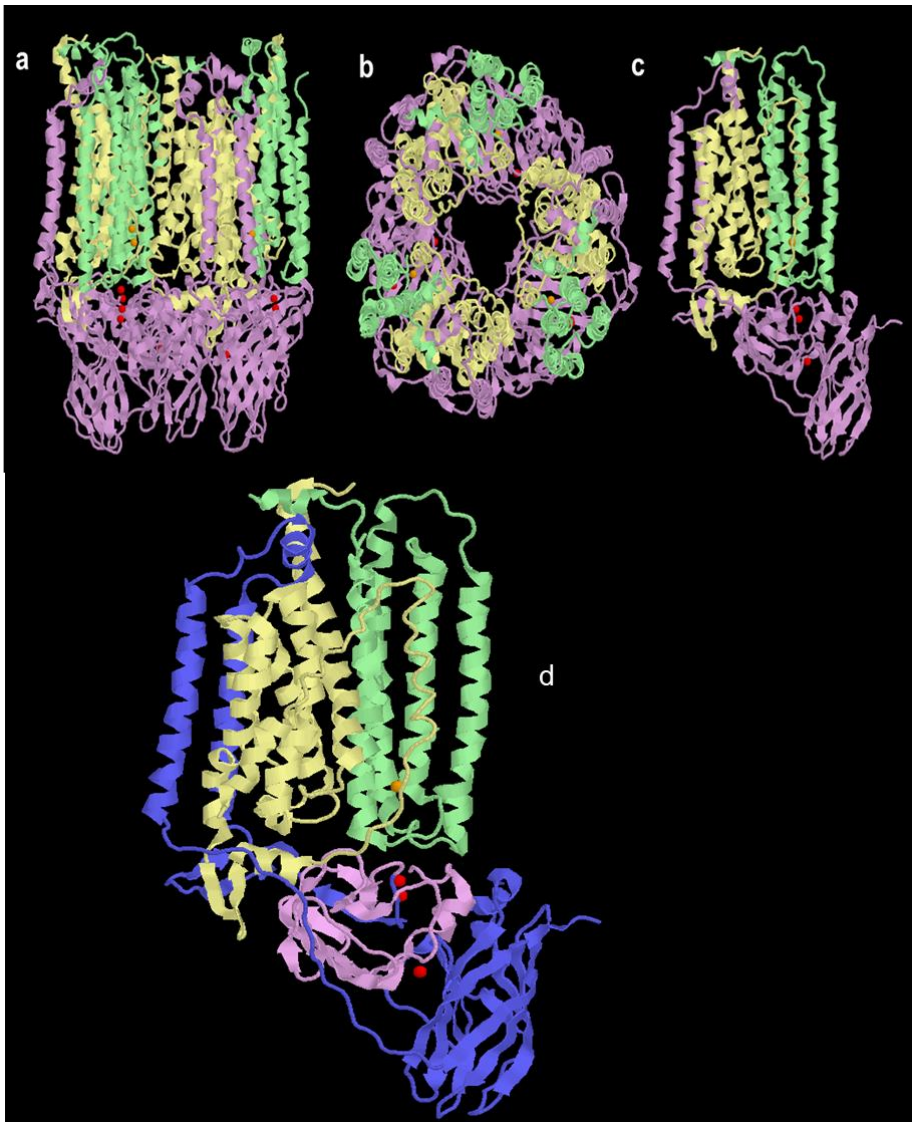


Figure 6.1 Structure of particulate methane monooxygenase pMMO.

The β-subunit (PmoA), α-subunit (PmoB), and γ-subunit (PmoC) are coloured lilac, yellow, and green, respectively. Metal atoms shown as spheres, copper red, and zinc orange, (a) $(\alpha\beta\gamma)_3$ enzyme complex; (b) view looking down on (a) from above; (c) one promoter showing the mononuclear and dinuclear copper and zinc centres (Smith and Murrell, 2010). (d) One pMMO protomer as in c showing the part of PmoB that is lost in the mutants in blue.

The fact that the selenite reducing activity in the cytoplasm is not removed when all the proteins in the fraction are digested indicates that the reducing agent that is immediately responsible for selenite reduction in this fraction is not a protein (Figs 5.5 and 5.7). This led to the question: how can the pMMO be indirectly responsible for reduction of selenite?

The unique step of the CH_4 oxidation pathway is the conversion of methane to methanol by methane monooxygenase (MMO) from methanotrophic bacteria. Methanotrophs oxidize methane to methanol via two important enzymes; particulate methane monooxygenase and soluble methane monooxygenase by incorporation of an oxygen atom across the C-H bond to produce methanol (Dalton, 1980; Feig & Lippard, 1994; Lipscomb, 1994; Wallar& Lipscomb, 1996; Merkx *et al*., 2001) as shown in Fig 6.2.

Methanotrophs, like other bacteria, have many metabolic reactions that require the reducing equivalents made available by their central metabolic pathways. These include the ability of certain methanotrophs to reduce Cr (VI), and there a large number reduction reaction that happen in any living cell, so inside the methanotrophs it needs electrons to perform these large number of reduction reaction. It is proposed that the effect of interrupting one of the copies of the pMMO operon on reduction of selenite is that there is a reduced rate of methane oxidation that leads to a smaller surplus of reducing power in the cell, so that most of the electrons are consumed by other essential reactions in the cell and fewer are available for reduction of selenite (Fig 6.2). Consistent with this hypothesis, reduction of selenite could be restored by supplying formate, which presumably made electrons available via formate dehydrogenase (Fig 4.10).

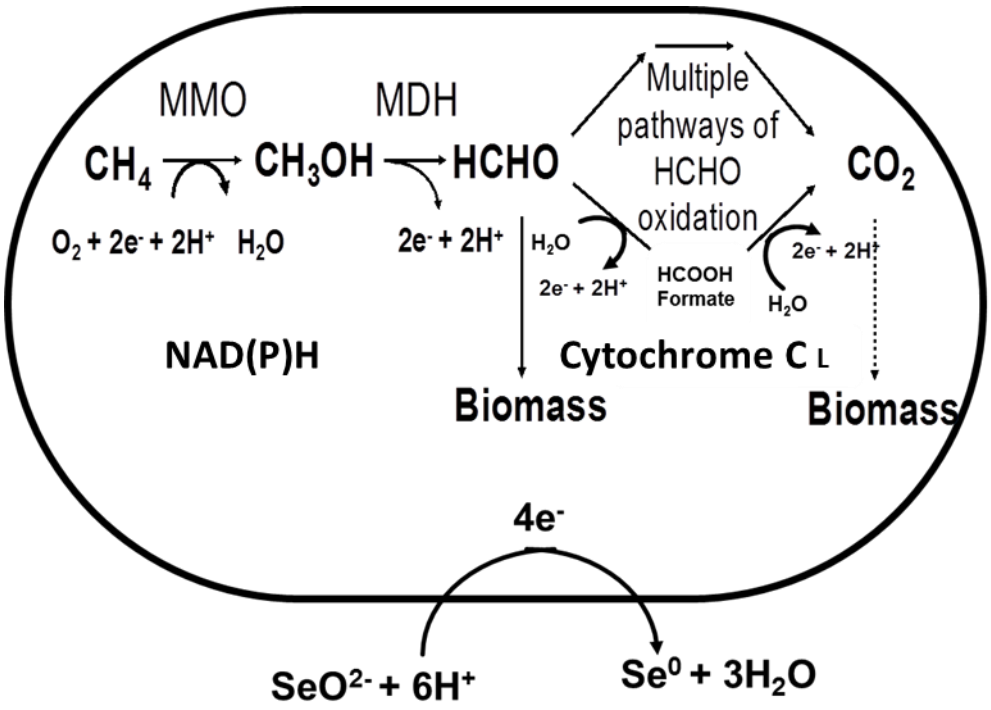


Figure 6.2. Shows the pathway of methane oxidation and carbon fixation by *Mc. capsulatus (Bath)* and its proposed link to reduction of selenite.

The results reported in this study showed that the reduction of selenite by methane-oxidizing bacteria is not likely to be due to a protein, unless that protein is resistant to digestion by trypsin and the endogenous proteases of the cell. Such a protein would also need to be heat-resistant since the selenite-reducing capacity of cell extracts is not destroyed by heating (Abdurrahman Eswayah, personal communication). The possibility of a protease-resistant enzyme could be explored by bioinformatic analysis of the genome of *Ms. trichosporium* to identify genes encoding protease-resistant proteins, although the author is not aware of such software that is currently available. A wide range of other (non-protein) molecules of diverse molecular masses, including polysaccharides, lipids and small molecule, may conceivably be involved in reduction of selenite.

Pseudomonas selenitipraecipitans strain CA-5 is capable of reducing both SeO_3^{2-} and SeO_4^{2-} to Se^0 (Hunter & Manter 2009). Analyses of fractions from

this strain indicate the presence of two reductases that can reduce SeO_3^{2-} to Se^0 in the presence of NADPH and that (based upon proteomics analysis of mixed protein samples) may correspond to glutathione reductase and thioredoxin reductase, both of which are able to reduce SeO_3^{2-} to Se^0 when derived from other sources (Hunter 2014a). Similar zymography and proteomic analysis of fractions from *Rhizobium selenitireducens* suggest that a protein belonging to the old yellow enzyme (OYE) family of flavo proteins is capable of reducing SeO_3^{2-} to Se^0 using NADH as the electron donor (Hunter 2014b).

The possibility that glutathione is involved in reduction of selenite offers a possible explanation for the results reported in this thesis. Glutathione, which is a tripeptide that lacks lysine and arginine sidechains required for digestion by trypsin, could be present in cellular fractions and be responsible for selenite reduction in these and in the intact cells. Ganther (1971) studied the reaction of selenite with glutathione (GSH), the most abundant thiol found in the eukaryotic cells, the cyanobacteria, and the α, β and γ groups of the proteobacteria, He showed that the selenotrisulfide of glutathione (GS-Se-SG), which was later renamed selenodiglutathione, is a very good substrate for glutathione reductase with Km and Vmax values comparable with those of glutathione itself as described in this equation.

$$GS\text{-}Se\text{-}SG + NADPH \xrightarrow{\text{Glutathione reductase}} GSH + GS\text{-}Se^{-} + NADP+$$

In experiments about the kinetics of selenite reduction in cultures of the phototrophic proteobacterium *Rhodospirillum rubrum* has been observed that the rate of the reaction is decreased significantly when the organism synthesizes low levels of glutathione (La¨ uchli, A 1993).

Experiments with the *R. rubrum* showed that the rate of selenite reduction was decreased when bacteria synthesized lower than normal levels of glutathione, and in *Rhodobacter sphaeroides* and *Escherichia coli* the reaction was reported to induce glutathione reductase (Janine and Kurt, 2004). Consistent with this suggestion, the annotations of the *Ms. trichosporium* OB3b genome

sequence indicate the presence of genes for glutathione synthase, glutathione reductase, glutathione peroxidase and glutathione-S-transferase (www.uniprot.org). Other thiol compounds such as mycothiol and thioredoxin are possible candidates for selenite reduction as well, although the annotations of the *Ms. trichosporium* genome do not give any indication that they are produced in this organism.

Among other stress-related proteins that may be involved in detoxification of or resistance to selenite, the DNA binding protein from starved cells (dps) found as part of the stress response in *E. coli* and other bacteria (Calhoun and Kwon, 2010) does not have any significant homologues in *Ms. trichosporium*.

6.2. Conclusions

The results presented in this thesis have included development of a system for transposon mutagenesis of methanotrophs by using *mariner* transposon in pSAM_R1, to create a library of mutants of *Ms trichosporium* OB3b and a identify specific gene that is involved in remediation of selenite $(SeO_3)^{2-}$ in the methanotrophs.

The results in this study show a role for the enzyme pMMO the remediation and reduction of selenite $(SeO_3)^{2-}$, although the molecular species directly responsible for selenite reduction in the cytoplasmic faction has not been identified, though it is likely not a protein. Investigation of the physiology of selenite reduction in *Ms. trichosporium* OB3b showed that the selenite-reducing activity was greatest in the cytoplasmic fraction.

6.3 Suggestions for future work

To investigate whether the role of the two full copies of the pMMO operon is similar in the other well characterized methanotroph *Mc. capsulatus* (Bath), a

similar mutant of one of the copies of *pmoB* could be constructed by means of marker exchange mutagenesis. It would be interesting not only to investigate the reduction of selenite by this mutant, but also to see whether the reduction of Cr (VI) to Cr (III) was affected by the diminished supply of electrons in the mutant.

The transposon mutagenesis method developed here may be used to identify potentially any gene involved in a specific function in *Ms. trichosporium*. This may include, for example, investigation of genes responsible for controlling the growth rate of methanotrophs, and other genes involved in transformation of selenite, chromium (VI) and other toxic metals and metalloids. This work also provides proof of principle for apply transposon mutagenesis to investigate other types of methanotrophs including investigation of the origin of adaptation in extremophilic methanotrophs such as acidophiles, alkaliphiles and thermophiles.

From this study we know that the cytoplasm has the greatest proportion of selenite reducing activity in the *Ms. trichosoprium* OB3b cell, although a protein is not directly involved in the reduction. It would be interesting further to fractionate the cytoplasmic fraction to purify and characterize the active component, and to test the hypothesis that it may be glutathione. It may then be possible to transfer the genes needed to produce this active species into other bacterial systems that are not able to reduce selenite, to create new selenite-reducing biocatalysts that could be powered by a variety of feedstocks, such as waste biomass.

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Appendixes

Appendix 1 media preparation

1.1) Preparation of Nitrate Mineral Salts Medium (0.1mg/L $CUSO_4.5\ H_2O$)

Preparation of 1 x NMS Medium

- 100 ml 10 x NMS salts solution.
- 1 ml Sodium Molybdate solution
- 1 ml Trace Elements Solution.
- ml Fe EDTA solution.
- Autoclave at 15 psi for 15 minutes.

For NMS agar plates 1.5% bacteriological agar (wt/v) is added to the medium prior to autoclaving. When the NMS is cool enough to hold in the hand aseptically add 10 ml phosphate buffer. If this is done too early the phosphate will precipitate out.

10 x NMS salts solution

- 10 g KNO_3
- 10 g $MgSO_4\ .7H_2O$ (4.8g $MgSO_4$)
- 2 g $CaCI_2.2H_2O$
- Up to 1 L sdH_2O

FE EDTA solution

- 3.8 g Fe EDTA
- Up to 100 ml sdH_2O

Sodium Molybdate solution

- 0.5 g $NaMo0_4.2H_2O$
- Up to 1 L sdH_2O

Trace elements solution

- 0.5 g $FeSO_4.7H_2O$
- 0.4 g $ZnSO_4.7H_2O$
- 0.015 g H_3BO_3
- 0.05 g $CoCl_2.6H_2O$
- 0.25 g EDTA (di sodium salt)
- 0.02 g $MnCl_2.4H_2O$
- 0.01 g $NiCl_2.6H_2O$
- up to 1 L with sdH_2O
- 0.1 g $CuSO_4.5H_2O$

Dissolve the above in the specified order in distilled water and dilute to 250 ml.

Store in the dark (wrap around with Aluminium foil).

Phosphate Buffer

- 49.7 g Na_2HPO_4
- Up to 1 L sdH_2O
- 39 g KH2P O4

pH should be 6.8 without altering. Sterilise by autoclaving in 100 ml aliquots and store at room temp.

1.2) LB Broth

- 2 litres of sterile distilled water (SDW).
- 50g LB powder (Difco).
- Mix to dissolve
- Dispense in 10ml aliquots into 50ml universal bottles.
- Autoclave.

1.3) LB agar Medium

- Measure 6g agar into each of 5, 400ml bottles.
- To 2 litres of SDW add 50g LB powder (Difco) and mix to dissolve.
- Add 400ml of SDW and LB powder dissolved solution to each bottles and shake to mix.
- Autoclave.

1.4) SOC Broth

(Prepared immediately before use)

- 2 ml of filter-sterilized 20 % (w/v) glucose or 1 ml of filter-sterilized
- 2 M glucose
- SOB medium (autoclaved) to a final volume of 100 ml

Appendix 2 Buffer preparation

Preparation of buffers for genomic tip-20 protocol

B1 buffer

- 18.61 g $Na_2EDTA\text{-}2H_2O$ • 6.06 g Tris base • 800 ml distilled water. • 50 ml 10% Tween-20 solution (v/v) • 50 ml 10% Triton X-100 solution, (v/v) • Adjust the pH to 8.0 with HCl. • Up to 1 L sdH_2O

B2 buffer

- 286.59 g guanidine HCl • 700 ml sdH_2O • 200 ml 100% Tween-20 (v/v) • Up to 1 L sdH_2O

QBT buffer

- 43.83 g NaCl, • 10.46 g MOPS • 800 ml distilled water. • Adjust the pH to 7.0 with NaOH. • Add 150 ml pure isopropanol • 15 ml 10% Triton X-100 solution (v/v) • Up to 1 L sdH_2O

QC buffer

- 58.44 g NaCl, • 10.46 g MOPS (free acid) • 800 ml distilled water. • Adjust the pH to 7.0 with NaOH. • Add 150 ml isopropanol. • Up to 1 L sdH_2O

QF buffer

- 73.05 g NaCl • 6.06 g Tris base • 800 ml distilled water. • Adjust the pH to 8.5 with HCl. • Add 150 ml pure isopropanol. • Up to 1 L sdH_2O

Appendix 3 Antibiotic

Antibiotic of kanamycin (Km)

- Measure 10 mg Kanamycin
- One ml SDW
- Mix

Antibiotic of Ampicillin (Amp)

- Measure 200 mg Ampicillin
- One ml SDW
- Mix

Tris. EDTA (T.E)

EDTA (1)

- Measure 1.85g EDTA
- 10ml SDW
- Mix together

Tris (2)

- Measure 1.21g Tris
- 10ml SDW
- Mix together

- Measure PH (HCL to get PH = 8)
- From (1) used 0.2ml, from (2) 1ml. Mix and autoclave