Methanosarcina acetivorans is a methanogenic archaeon capable of utilizing monomethylamine (MMA), dimethylamine (DMA) and trimethylamine (TMA) for methanogenesis. The mtmP, mtbP, mttP1 and mttP2 genes were predicted to encode MMA, DMA, TMA and TMA permeases, respectively, based on their genomic context and bioinformatic analysis. We investigated the roles of these predicted permeases in growth on methylamines. The ΔmtmP, ΔmtbP, and ΔmttP1/ΔmttP2 deletion strains were created using a markerless genetic exchange method. Unaltered growth phenotypes of the mutants on their respective methylamines, except for an extended lag phase in the ΔmtmP mutant, suggest the respective substrates are still transported in the absence of the predicted permeases. These results could be due to broad substrate specificity of the permeases or due to alternative permease activity when grown in pure culture under laboratory conditions of substrate concentration. The ΔmtmP/ΔmtbP and ΔmtbP/ΔmttP1/ΔmttP2 deletion strains show some defect in DMA-dependent growth suggesting cross-reactivity between methylamine permeases.
EXAMINATION OF PUTATIVE METHYLAMINE PERMEASE GENES IN
*Methanosarcina acetivorans* C2A

A Thesis

Submitted to the
Faculty of Miami University
in partial fulfillment of
the requirements for the degree of
Master of Science
Department of Microbiology
by
Subash Dhungana
Miami University
Oxford, Ohio
2014

Advisor___________________________
Donald J. Ferguson

Reader____________________________
Gary R. Janssen

Committee Member____________________________
Annette Bollmann
TABLE OF CONTENTS

List of Tables ........................................................................................................... P. iii
List of Figures ........................................................................................................... P. iv-v
Introduction ............................................................................................................ P. 1-14
Materials and Methods ........................................................................................ P. 15-27
Results .................................................................................................................... P. 28-70
Discussion ............................................................................................................. P. 71-82
References ............................................................................................................. P. 83-86
LIST OF TABLES

Table 1. List of strains and plasmids used in this work………………………… P. 15
Table 2. List of primers used in this work………………………………………… P. 23
Table 3. List of knocked out genes with RE used for Southern hybridization along with predicted band size for parental strain and knockout strains…….. P. 27
LIST OF FIGURES

Figure 1. Methanogenesis pathways and the Wolfe cycle of hydrogenotrophic methanogenesis. ................................................................. P. 9

Figure 2. Schematic diagram of CoM methylation pathways from different methylotrophic substrates using analogous enzymes......................... P. 11

Figure 3. Gene arrangement of different methanogenic operons.......................... P. 13

Figure 4. Predicted transmembrane helices of putative methylamine permeases... P. 37

Figure 5. Cladogram of the putative MMA permease and related proteins........ P. 39

Figure 6. Cladogram of the putative DMA permease and related proteins........ P. 41

Figure 7. Cladogram of the two putative TMA permeases and related proteins... P. 43

Figure 8. Screening and confirmation of the mtmP deletion from WWM73 using the markerless genetic exchange method.......................... P. 45

Figure 9. Growth curves of DJF17 (ΔmtmP) and WWM73 on different substrates.. P. 47

Figure 10. Screening and confirmation of the mtbP deletion from WWM73....... P. 49

Figure 11. Screening and confirmation of the mttP2 deletion from WWM73....... P. 51

Figure 12. Southern hybridizations to confirm deletion of M. acetivorans mttP1/mttP2 genes in DJF37............................................................... P. 53

Figure 13. Screening and confirmation of the mttP1 deletion from WWM73...... P. 55

Figure 14. Growth curves of DJF22 (ΔmtbP) and WWM73 on different substrates.. P. 57

Figure 15. Growth curves of DJF37 (ΔmttP1/ΔmttP2) and WWM73 on different substrates................................................................. P. 59

Figure 16. Screening and confirmation of the mtbP gene deletion from DJF17 (ΔmtmP)................................................................. P. 61

Figure 17. Growth curves of DJF52 (ΔmtmP/ΔmtbP) and WWM73 grown on different substrates............................................................... P. 63

Figure 18. Screening and confirmation of the mtbP deletion from DJF37 (ΔmttP1/ΔmttP2)........................................................................ P. 66

Figure 19. Growth curves of DJF51 (ΔmtbP/ΔmttP1/ΔmttP2) and WWM73 grown on different substrates............................................................... P. 68
Figure 20. Structure of monomethylamine, dimethylamine, trimethylamine and glycine betaine................................................................. P. 79

Figure 21. Structure of GABA and its analogues.................................................P. 81
Acknowledgments

I would like to thank Dr. D. J. Ferguson for his continuous support and guidance throughout this work. I would also like to thank my committee members Dr. Annette Bollmann and Dr. Gary Janssen for the helpful comments and suggestions. I’d like to extend special thank you to Dr. Janssen for reading and reviewing my thesis.

I would also like to thank my lab mates Tomislav and Dinesh.

I would like to thank my dad, mom and two sisters who provided me encouragement and support. They are also the inspiration for the hard work.

Finally, I would like to thank all the friends in Oxford who made my stay memorable.
I. INTRODUCTION

_Eukarya, Bacteria, and Archaea_ are the three domains of cellular life on earth. Based on 16S ribosomal rRNA and a conserved genomic core, three phyla of the archaeal domain, _Crenarchaeota, Euryarcharyota_ and _Thaumarchaeota_, have been described (Brochier-Armanet _et al._, 2008). With complete genomic analyses of these groups now available, the _Crenarchaeota_ consist of hyperthermophilic archaea while the _Thaumarchaeota_ contain the mesophilic archaea which branched out recently from the _Crenarchaeota_. The _Euryarcharyota_ are a phylogenetically diverse group of archaea which consist of methanogens, halophilic, thermophilic and sulfate-reducing organisms (Brochier-Armanet _et al._, 2008). At present, seven orders of methanogens have been described in the _Euryarcharyota_, namely _Methanopyrales, Methanococcales, Methanobacteriales, Methanomicrobiales, Methanosarcinales, Methanocellales_, and the recently proposed _Methanoplasmatales_ (Nonoh _et al._, 2012).

All of these methanogens are strictly anaerobic and produce methane as a catabolic end product. The process of biological methane production, known as methanogenesis, is an anaerobic respiration responsible for production of around one billion tons of methane per year and is a significant contributor to global warming (Costa and Leigh, 2014; Hook _et al._, 2010). Although methane is present in trace amounts (i.e., 1774±1.8 parts per billion (ppb) global concentration in 2010, with an increase of 11 ppb since 1998), it is still considered the most potent global warming gas after CO₂ because it is 25 times more potent than CO₂ and has a 12-year atmospheric lifetime (Hook _et al._, 2010). Methane increases the ozone in a greenhouse-affected atmosphere and increases atmospheric water vapor, thus accounting for a 70% increase in the radioactive force of this gas (Hook _et al._, 2010).

On the basis of the carbon source used, methanogens are classified into two metabolic groups: hydrogenotrophs and methylotrophs. The orders _Methanosarcinales_ and, recently proposed, _Methanoplasmatales_ are included in methylotrophs which are characterized by their ability to utilize the methyl group from acetate and other methyl-containing compounds like methylamines and methanol (Nonoh _et al._, 2012; Costa and Leigh, 2014). However, some of the methanotrophs are also able to reduce CO₂ to CH₄. The rest of the five orders are included in the hydrogenotrophs that reduce CO₂ to CH₄;
most use H\textsubscript{2} as an electron donor but a few can use formate, 2-propanol, 2-butanol, cyclopentanol, and ethanol as an electron source (Hedderich and Whitman, 2013). The major difference between hydrogenotrophs and methylotrophs lies in their mechanism of energy conservation during methanogenesis. In the case of hydrogenotrophs, electron bifurcation is an integral part of energy coupling in which energy is conserved when reduction of higher potential substrate (heterodisulfide reduction) is coupled with reduction of lower potential substrate (CO\textsubscript{2} reduction). The exact mechanistic model of electron bifurcation is unknown, however, electron bifurcation revolves around a heterodisulfide reductase (Hdr) complex as described in the proposed Wolfe cycle (Figure 1B). Reduction of CO\textsubscript{2} requires additional electron supplement apart from stoichiometric electron supply, which is accomplished by a membrane bound, energy-converting hydrogenase that consumes chemiosmotic membrane potential and supplies electrons through ferredoxin (Fd) reduction. In contrast, methylotrophs do not require electron bifurcation for CO\textsubscript{2} reduction when CO\textsubscript{2} is the carbon source. Unlike hydrogenotrophic methanogenesis, all of the electrons required for CO\textsubscript{2} reduction are supplied by a hydrogenase consuming chemiosmotic membrane potential. Heterodisulfide reduction is coupled to energy conservation where a membrane bound hydrogenase generates chemiosmotic membrane potential that transports H\textsuperscript{+} through a membrane bound electron transport chain (Costa and Leigh, 2014; Hedderich and Whitman, 2013).

\textit{Methanosarcina acetivorans}

\textit{Methanosarcina acetivorans} is classified under the order \textit{Methanosarcinales} and family \textit{Methanosarcinaceae} (Sowers et al., 1984). \textit{M. acetivorans} C2A is a wild-type strain isolated from a methane-evolving acetotrophic marine sediment. \textit{M. acetivorans} stains gram negative and appears as an irregularly shaped coccus 1.9 ± 0.2 \textmu m in diameter with fimbria at the cell surface, but are non-motile. It is strictly anaerobic and grows at an optimum temperature of 35-40°C and an optimum pH range of 6.5 to 7 (Sowers et al., 1984). It grows as aggregates of cells in low salt growth medium (salt < 0.4 M) but as dispersed cells in salt concentrations at or above 0.4 M. Structural studies of \textit{M. acetivorans} C2A cells grown in different salt concentrations revealed the presence
of a methanochondroitin outer layer when grown in low salt but this layer was absent with just S-layer protein above the cell membrane when grown in high salt (Sowers et al., 1993).

*M. acetivorans* C2A contains the largest archaeal genome known to date (5,751,492 base pairs [bp]) with a mol G+C content of 42.7 mol % and contains nearly 200 genes (5% of the predicted open reading frames) related to methanogenesis. These genes are related to utilization of acetate, carbon monoxide (CO) and methylated compounds such as methanol and methylamines. Most of these genes are present in multiple copies, reflecting the importance of methanogenesis, which is the only means of energy conservation in this organism. The presence of multiple copies of these genes indicate that *M. acetivorans* C2A is specialized in the pathways that utilize acetate (acetoclastic pathway), carbon monoxide (CO; carboxidotrophic pathway) and other methyl containing compounds (methylotrophic pathway) (Galagan et al., 2002; Matschiavelli et al., 2012). The carboxidotrophic pathway is unique to *M. acetivorans* because CO is used to produce acetate with its unique set of enzymes, unlike other freshwater methanogens which convert CO to CO₂ and proceed through the hydrogenotrophic pathway (Hedderich and Whitman, 2014). The fact that it is isolated from acetate- and methylamine-rich marine environments and the presence of multiple copies of genes for acetoclastic and methanotrophic pathways suggests *M. acetivorans* C2A evolved to adapt to such habitats. Unlike many *Methanosarcina* species, *M. acetivorans* C2A cannot use H₂ to reduce CO₂ (hydrogenotrophic pathway) because it lacks the hydrogenase required for the oxidation of H₂ to provide electrons for CO₂ reduction. It has been reported that two different hydrogenase genes are present in *M. acetivorans* but it appears that they do not function to oxidize H₂ (Galagan et al., 2002). The availability of the genome sequence, the versatility of its growth in different substrates and the development of genetically modified strains and molecular tools makes *M. acetivorans* C2A a model organism for molecular characterization and genetic engineering of methanogens. The ability of *M. acetivorans* to grow as single cells (Sowers et al., 1993) makes mutant selection and screening for genetic studies possible. Currently, the tools available for genetic manipulation of *M. acetivorans* C2A include targeted deletions, introduction of genetic material into cells by PEG-mediated
transformation, plasmids for expression of manipulated genes, and a few selectable markers for genetic screening (Buan et al., 2011, Kohler and Metcalf, 2012). The relative ease of deleting specific genes and screening the resulting phenotypes in *M. acetivorans* makes it an important species for characterization of genes of unknown function.

*M. acetivorans* WWM73 is derived from wild-type *M. acetivorans* C2A strain and optimized for genetic manipulation in the laboratory. The hypoxanthine phosphoribosyltransferase (*hpt*) gene has been deleted in WWM73 in order to use it as a counterselection marker (Pritchett et al., 2004). The ΦC31 site-specific recombinase gene (*int*), *tetR* gene and the ΦC31 *attP* site have been inserted at the *hpt* locus in this strain. The *attP* site is utilized to complement a gene by site-specific recombination with a plasmid carrying an *attB* site (Guss et al., 2008).

**The methylotrophic pathway of methanogenesis in *M. acetivorans***

The methylotrophic pathway utilizes different methylated compounds such as monomethylamine (MMA), dimethylamine (DMA), trimethylamine (TMA), tetramethylammonium (QMA), methanol, methylthiols, and methylsulfide. In each methylotrophic pathway, a methyl group is transferred from one of these methylated compounds in a series of reactions to coenzyme M (CoM), generating methyl-CoM, which is ultimately reduced to methane. The two electrons required for the final reduction step is supplied by oxidation of an additional methyl group to CO$_2$. Six electrons are produced during oxidation of one molecule of a methylated compound to CO$_2$, which is enough to reduce the methyl group of three molecules of methyl-CoM to methane using two electrons per reduction (Figure 1A). However, methylated compounds could be completely reduced to methane when H$_2$ (electron donor) is supplied (Hedderich and Whitman, 2013). Production of methane could be regarded as a waste byproduct to the cell. However, heterodisulfide formed in the same reaction is energetically very important for the cell because heterodisulfide reduction is coupled with energy conservation (Deppenmeier *et al.* 2004), where CoM-H-H-CoB can be regarded as a terminal electron acceptor and H$_2$ or coenzyme F$_{430}$H$_2$ as an electron donor.
depending on the methylated compound in the respiratory chain of methylotrophs (Hedderich and Whitman, 2013).

**Activation of methylated compounds**

The methylotrophic pathway begins with the transfer of a methyl group from the methylated compound onto coenzyme B. Each methylated compound has a specific methyltransferase system that carries out methyl transfer to coenzyme B through an analogous pathway (Figure 2). Each methyltrasferase system consists of a substrate specific corrinoid protein, MtxC, and two methyltrasferases, MT1 and MT2. The MT1 of methanol (MtaB), monomethylamine (MtmB), dimethylamine (MtbB) and trimethylamine (MttB) transfer a methyl group from their specific substrate onto a substrate-specific corrinoid protein (MtxC). The second methyltransferase, MT2, of methanol (MtaA), monomethylamine (MtbA), dimethylamine (MtbA) and trimethylamine (MtaA or MtbA) transfers the methyl group from their cognate corrinoid protein onto coenzyme M (Burke and Krzycki, 1998; Ferguson et al., 2000; Ferguson and Krzycki, 1997; Ferguson et al., 1996; Hedderich and Whitman, 2013). All of these pathways start with different substrates and different enzymes but all of them end up with a common end product, methyl-coenzyme M, which is reduced with another coenzyme, called coenzyme B, to form the heterodisulfide CoM-S-S-CoB and methane. This reaction is catalyzed by methyl-coenzyme M reductase (Mcr), which is a unique enzyme to methanogenesis and is often used for characterizing methanogens (DiMarco et al., 1990).

**Arrangement of the genes involved in the methylamine catabolic pathways**

The genes encoding the methyltransferases and specific corrinoid proteins for MMA, DMA and TMA are arranged in seven different operons in *M. acetivorans*. There are two MMA catabolic operons, three DMA catabolic operons and two operons with both DMA and TMA catabolic genes. Out of these multiple catabolic operons, one each of MMA and DMA catabolic operons with their specific methyltransferases and corrinoid proteins, have putative permease genes whereas two operons with DMA and TMA catabolic genes have putative permease genes downstream of methyltransferases and corrinoid proteins (Figure 3). Each of the four operons, with an uncharacterized
gene following the methyltransferases and corrinoid protein encoding genes, are highly conserved among different species of the *Methanosarcinales* and are located with methylamine catabolic operons. Bioinformatic analysis of the proteins encoded by these genes indicates that these are transmembrane proteins and are closely related to known permeases. The putative permease genes *mtmP*, *mtbP*, *mttP1* and *mttP2* have been identified on the basis of their proximity to other related genes involved in methanogenesis. Bioinformatic data shows the putative MMA and DMA permeases, *mtmP* and *mtbP*, are closely related to the amino acid/polyamine/organocation (APC) family of cationic amine permeases (Burke *et al.*, 1998) and the putative TMA permeases are closely related to the choline uptake transporter (LicB-T) family (Paul *et al.*, 2000). Furthermore, in a proteomics study of *Methanococcoides burtonii*, the TMA methyltransferases and their corrinoid proteins were found in the insoluble fraction of protein, along with another predicted TMA permease (Williams *et al.*, 2010). The arrangement of predicted permeases within their respective substrate-specific operons might help facilitate TMA catabolism by coupling substrate uptake with methyl transfer by forming a membrane-associated complex of all the catabolic proteins and the putative permease.

One or two short and highly conserved open reading frames (Orfs) are located adjacent to the predicted MMA permease gene, *mtmP*, and two predicted TMA permease genes, *mttP1* and *mttP2*, have been observed across species of *Methanosarcinales*. The function of these Orfs is still under question. In *Methanococcoides burtonii*, the Orf adjacent to *mttP1* and *mttP2* was expressed and found in abundance when grown in TMA. Therefore, these Orfs might be important in mediating interaction between predicted permeases and their respective methyltransferase and corrinoid protein (Williams *et al.*, 2010).

**Amino acid-Polyamine-Organocation (APC) super family transporter**

The predicted *mtmP* and *mtbP* permeases of *Methanosarcina acetivorans* are classified within the APC family of transporters, one member of the APC superfamily of transporters. APC transporters are the largest secondary carrier superfamily and the APC superfamily has recently been designated the largest superfamily, replacing the major
facilitator superfamily (MFS), based on 3D structural similarity. The APC and MFS superfamilies have evolved from a common ancestor; however, both have undergone major structural changes to give a 3D structure characteristic of their respective superfamily. The APC superfamily is characterized by the presence of duplicated repeats of five transmembrane α-helical segments (TMSs). One to three additional TMS may be present at their N- or C-termini (Wong et al., 2012). Five transmembrane repeats are intertwined to form a distinct three-dimensional structure to facilitate the mechanism of secondary solute transport. Recently, a structural biology approach has united sequentially diverse amino acid transporters that bind to structurally diverse substrates and have different coupling modes by displaying common overall folds (Schwelkhard and Ziegler, 2012). The common fold is known as the APC fold and all the proteins are classified under the APC super family which displays a common general mechanism of transport despite very low sequence identity and coupling modes between the protein families (Schwelkhard and Ziegler, 2012). Based on 3D structure, eleven families have been proposed which includes (1) APC (TC No. 2.A.3), (2) amino acid/auxin permease (AAAP; TC No. 2.A.18), (3) alanine or glycine:cation symporter (AGCS; TC No. 2.A.25), (4) cation chloride co-transporter (CCC; TC No. 2.A.30), (5) hydroxy/aromatic amino acid permease (HAAAP; TC No. 2.A.42), (6) betaine/carnitine/choline transporter (BCCT; TC No. 2.A.15), (7) solute:sodium symporter (SSS; TC No. 2.A.21), (8) neurotransmitter:sodium symporter (NSS; TC No. 2.A.22), (9) nucleobase:cation symporter-1 (NCS1; TC No. 2.A.39), (10) nucleobase:cation symporter-2 (NCS2; TC No. 2.A.40), and (11) sulfate permease (SulP; TC No. 2.A.53) (Wong et al., 2012).

Protein members, descriptions and references for these families are available at the transporter classification database (www.tcdb.org).

The Drug/Metabolite Transporter (DMT) Superfamily

Based on multiple sequence alignments, the predicted TMA permeases are closely related to the choline uptake transporter (LicB-T) family that is classified within the drug/metabolite transporter (DMT) superfamily at the transporter classification database (www.tcdb.org). The choline uptake transporter family and APC family transporters have been predicted to have a common transport mechanism by
solute:proton symport: \( \text{Solute (out)} + n \text{H}^+ \text{ (out)} \rightarrow \text{Solute (in)} + n \text{H}^+ \text{ (in)} \), and both families have a similar transmembrane topology of a characteristic 10 TMS.

The Goals of My Thesis Work

Most of the enzymes of methylamine methanogenic pathways have been characterized and the utilization of different methylamines by \( M. \text{acetivorans} \) is well established. However, the proteins involved in the transport of these substrates into the cell are still largely unknown. The characterization of the methylamine permeases will help us to further understand the mechanism of substrate utilization in these pathways. The overall aim of this project is to identify and characterize genes encoding putative monomethylamine (\( \text{mtmP} \)), dimethylamine (\( \text{mhbP} \)) and trimethylamine (\( \text{mttP1} \) and \( \text{mttP2} \)) permeases in \( M. \text{acetivorans} \). In order to achieve this goal, the predicted methylamine permease genes are deleted from the genome using established genetic exchange methods, based on homologous recombination, that were developed for \( M. \text{acetivorans} \) (Pritchett et al., 2004); after deletion of the putative permease genes, the resulting mutant strains are analyzed for their growth phenotypes using a variety of methylamine substrates.
**Figure 1.** Methanogenesis pathways and the Wolfe cycle of hydrogenotrophic methanogenesis. **A. Methanogenesis Pathways.** Hydrogenotrophic (solid black lines), methylotrophic (dashed black lines), and aceticlastic (grey lines) pathways of methanogenesis all share steps of methyl-S-CoM and CoM-S-S-CoB reduction. In methylotrophic methanogenesis, four methylated compounds enter at the level of methyl-S-CoM and one is oxidized to generate the electrons needed to reduce the remaining three to methane. In aceticlastic methanogenesis, the methyl carbon of acetate enters at the level of methyl-H₄MPT and uses the electrons produced by oxidation of the carbonyl carbon to produce methane. The number of electrons transferred in each redox reaction is shown next to the appropriate arrows. The energy yield of each reaction ($\Delta G^\circ$, kJ/mol) is shown in numbers in blue. The reactions important to energy conservation or energy depletion is in bold and italicized. MFR, methanofuran; H₄MPT, tetrahydromethanopterin; HS-CoM, coenzyme M; HS-CoB, coenzyme B; F₄₂₀H₂, reduced form of the electron carrying coenzyme F₄₂₀. **B. The Wolfe cycle of hydrogenotrophic methanogenesis.** The heterodisulfide reductase (Hdr) complex catalyses reduction of heterodisulfide CoM-S-S-CoB and ferridoxin (Fd) where electron bifurcation takes place. Reduction of carbon dioxide to formyl-MFR uses reduced Fd making the pathway cyclic. Intermediate reduction step in the pathway is carried out by a flow of electrons through F₄₂₀ (Adopted from Costa and Leigh, 2014)
**Figure 2.** Schematic diagram of CoM methylation pathways from different methylo trophic substrates using analogous enzymes. Methyl group transfer from TMA, DMA, MMA, and methanol to CoM via different sets of analogous methyltransferase and corrinoid protein. (Adopted from Ferguson *et al.*, 2000)
**Figure 3.** Arrangement of genes involved in the methylamine catabolic pathways of *M. acetivorans*. **A.** MMA methanogenic operon with genes encoding MMA-specific corrinoid (*mtmC*) and methyltransferase (*mtmB*) proteins, and a putative permease (*mtmP*; MA0143) followed by two Orfs (orf1; MA0144 and orf2; MA0145). **B.** DMA methanogenic operon with genes encoding DMA-specific corrinoid (*mtbC*) and methyltransferase (*mtbB*) proteins, followed by a predicted DMA permease (*mtbP*; MA2426). **C.** TMA methanogenic operon with genes encoding DMA-specific corrinoid (*mtbC*), TMA-specific methyltransferase (*mttB*), TMA-specific corrinoid (*mttC*), and putative TMA permease (*mttP1*; MA0530) proteins, followed by an Orf (orf1; MA0531) that is predicted to have a role in the interaction between the permease and the corrinoid protein-methyltransferase complex needed for efficient TMA-specific methanogenesis. **D.** The second TMA methanogenic operon with genes encoding DMA-specific methyltransferase and corrinoid proteins, TMA-specific methyl transferase and corrinoid proteins followed by two Orfs (orf1; MA0930 and orf2; MA0928) that are separated by a predicted permease (*mttP2*; MA0929).
II. MATERIALS AND METHODS

II.1 Strains and plasmids

Table 1. List of strains and plasmids used in this work.

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methanosarcina acetivorans strains and derivatives</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2A</td>
<td>Stock DSM2834</td>
<td>Wild-type</td>
</tr>
<tr>
<td>WWM73</td>
<td>C2A with Δhtp::(P&lt;sub&gt;mtbR&lt;/sub&gt;PhiC31 int attP) genotype modification</td>
<td>Guess et al. (2008)</td>
</tr>
<tr>
<td>DJF17</td>
<td>mtmP deletion from WWM73</td>
<td>This work</td>
</tr>
<tr>
<td>DJF18</td>
<td>DJF17 complemented with plasmid expressing mtmP</td>
<td></td>
</tr>
<tr>
<td>DJF22</td>
<td>mtbP deletion from WWM73</td>
<td>This work</td>
</tr>
<tr>
<td>DJF27</td>
<td>mttP&lt;sub&gt;1&lt;/sub&gt; deletion from WWM73</td>
<td>This work</td>
</tr>
<tr>
<td>DJF32</td>
<td>mttP&lt;sub&gt;2&lt;/sub&gt; deletion from WWM73</td>
<td>This work</td>
</tr>
<tr>
<td>DJF37</td>
<td>mttP&lt;sub&gt;1&lt;/sub&gt; deletion from DJF32</td>
<td>This work</td>
</tr>
<tr>
<td>DJF51</td>
<td>mtbP deletion from DJF37</td>
<td>This work</td>
</tr>
<tr>
<td>DJF52</td>
<td>mtbP deletion from DJF17</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Bacterial Strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> EC100</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMP44</td>
<td>Plasmid with oriR6K, bla, pac, hpt genotype</td>
<td>Pritchett et al. (2004)</td>
</tr>
<tr>
<td>pTL1</td>
<td>mtmP cloned into pMP44</td>
<td>This work</td>
</tr>
<tr>
<td>pTG1</td>
<td>mttP&lt;sub&gt;1&lt;/sub&gt; cloned into pMP44</td>
<td>This work</td>
</tr>
<tr>
<td>pTO1</td>
<td>mttP&lt;sub&gt;2&lt;/sub&gt; cloned into pMP44</td>
<td>This work</td>
</tr>
<tr>
<td>pTS1</td>
<td>mtbP cloned into pMP44</td>
<td>This work</td>
</tr>
</tbody>
</table>
II.2 Growth medium and additives

*Escherichia coli* cultures were grown in Luria Bertani (LB) medium. Premixed LB broth (Peptone from casein 1.0% (w/v), yeast extract 0.5% (w/v), NaCl 1% (w/v) from EMD Chemicals Inc. was used. 2% agar (w/v) was added to make LB agar plates. Filter sterilized (0.2µm pore size) ampicillin was added at a final concentration of 100µg/mL, from the stock of 100mg/mL, to autoclaved and cooled LB medium.

*M. acetivorans* strains were grown as single cells in High Salt (HS) broth medium at 37 °C. HS medium was prepared in strictly anaerobic Mix A (400mM NaCl, 45 mM NaHCO₃, 13 mM KCl, 1% resazurin, trace elements (1.6 µM H₃BO₃, 0.4 µM CuSO₄, 4 µM NiCl₂, 58 µM Ni(CH₂CO₂H)₃, 4 µM Na₂MoO₄, 11 µM Na₂SeO₃, 3 µM Na₂WO₄, 3 µM ZnSO₄, 4 µM CoCl₂, 20 µM Fe(NH₄)₂(SO₄)₂, 6 µM MnCl₂/MnSO₄) and Mix B (54 mM MgCl₂, 2 mM CaCl₂) was prepared separately and then transferred into a manifold where these mixes undergo 10 min of degassing with the stream of H₂/CO₂ (80%/20%). Both mixes were closed with stoppers to prevent O₂ from entering into the flask and taken into the anaerobic chamber (glove bag) (Coy Laboratories, Inc., Grass Lake, MI). Mix A and Mix B were mixed inside the glove bag and 5 mM KH₂PO₄, 19 mM NH₄Cl, 2.8 mM cysteine-HCl, 0.4 mM Na₂S were added (final concentrations). The medium was dispensed as 10 mL into 27 mL tubes and 50 mL into 150mL serum bottles. Once filled, the tubes and bottles were capped with a stopper and taken out of the glove bag. Plasticware used in the glove bag was kept in glove bag for at least 3 days to remove all the absorbed oxygen. Glassware was placed in glove bag for at least one day prior to using it for the same reason. Tubes and serum vials were crimped with aluminum caps and the headspace was exchanged four times under H₂/CO₂ (80%/20%) for 1 min evacuation cycles and pressurized to 2 psi. The medium-filled tubes were then autoclaved, cooled, and shaken until the precipitant re-dissolved. Substrates (2M-4M) and vitamins (1000X; 113 µM Folic acid, 242 Thiocotic acid, 729 µM p-Aminobezoic Acid, 812 µM Nicotinic acid, 486 µM Pyridoxine HCl, 266 µM Riboflavin, 296 µM Thimine HCl, 204 µM Biotin, 419 µM Ca Pantothenate, 37 µM Hydroxocobalamin HCl) were prepared and sterilized separately and added at the time of inoculation using a syringe. The final concentrations of substrates added to the tubes were as follows: 20 to 40 mM TMA (HST), DMA (HSD), MMA (HSMa) or 125mM methanol-40mM acetate
(HSMA). Each type of medium contained only one substrate except HSMA, which contained both methanol and acetate, as indicated above.

II.3 Sterilization:
All growth media were vapor sterilized at 121°C for 40 minutes. Other solutions were also vapor sterilized unless it is temperature sensitive. Temperature sensitive solutions like ampicillin, 8-aza-2,6-diaminopurine (8-ADP), and vitamins were filter sterilized using 0.2µm pore size filter.

II.4 M. acetivorans plate culture
Agar (1.5% w/v) was added anaerobically to HS medium in the glove bag, which was capped with a stopper, crimped with aluminum cap, and autoclaved. Substrates, vitamins, and additional additives like antibiotics (puromycin) and 8-ADP were then added using a syringe. Puromycin (2µg/ml (final concentration) was added from 0.4 mg/mL (200X) stock. 2 µg/mL (final concentration) of 8-ADP was added from 2 mg/mL (100X) stock. Media was taken into the anaerobic glove bag and poured into Petri dishes before the agar solidified.

II.5 Liquid culture of E. coli
Liquid cultures (2-5 mL) of E. coli were inoculated from glycerol stocks (section II.8) or a single colony from LB agar plate medium. Appropriate antibiotics were added and the culture was grown for 17 hours at 37 °C with shaking at 200 rpm in a culture tube.

II.6 Liquid culture of M. acetivorans:
A fresh tube with appropriate substrates and antibiotics was inoculated with 0.1 mL inoculum from a grown culture using a 1 mL syringe with 22 gauge needle. For a liquid culture from agar plate culture, an isolated colony was picked into a 1 mL syringe without attaching a needle (as described in Buan et. al. (2011)). Once a colony from agar was picked with the syringe, a needle was attached, and the culture tube was inoculated by flushing the syringe a few times with the liquid medium.
II.7 Determination of cell density:

Optical density at wavelength of 600nM was measured to determine cell density of liquid cultures using spectrophotometer.

II.8 Storage of *M. acetivorans* and *E. coli*

Both *M. acetivorans* and *E. coli* were kept on agar media plates for up to 4 weeks after growth. Liquid cultures of *M. acetivorans* were also stored for up to 4 weeks and transferred to new tubes. Glycerol stocks, as described below, were prepared for each strain for long term storage.

*M. acetivorans* long term storage and revival was performed as described by Whitman *et al.* (1986). 40 µL of 100% glycerol was added to 2 mL glass vial (Wheaton #224881). The vial was autoclaved and kept in the bag anaerobically for 3 days before use. 10 mL aliquots of a *M. acetivorans* culture was centrifuged for 10 min at 7000rpm. The pellet was resuspended in 1 mL of fresh media with substrate (HSMA). 160 µL of resuspended 10X concentrated culture was added to sterile vials with glycerol (= 20% final concentration glycerol), which was tightly capped and immediately transferred to -80 °C. In order to revive glycerol stocks, vials were quickly brought into the bag along with fresh culture tubes containing 10 mL of media. Once the stock culture is thawed, the entire volume of culture was inoculated into a tube with 10 mL of growth medium and incubated at 37 °C.

For *E. coli* glycerol stocks, liquid cultures were grown at 37 °C in a shaker until OD<sub>600</sub> > 2. 730 µL of densely grown liquid culture and 270 µL of 70% glycerol was mixed and immediately transferred to -80 °C for storage (glycerol concentration = 19%). For revival, a small aliquot of the frozen culture was removed with a micropipette tip or sterile inoculating loops and inoculated into fresh LB liquid medium or streaked onto LB agar.

II.9 Preparation and transformation of competent *E. coli*

*E. coli* EC100 chemically competent cells were prepared by a variation of Hanahan *et al.* (1991). EC100 cells were grown in 250 mL of SOB medium (0.5% (w/v)
yeast extract 2% (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 20 mM MgSO$_4$) at 20 °C to OD$_{600}$ of 0.3. The cells were centrifuged at 3000 rpm at 4 °C for 10 min. The pellets were resuspended in 10 mL of ice cold CCMB80 buffer (10mM potassium acetate; pH 7.0, 80mM CaCl$_2$, 20mM MnCl$_2$, 10mM MgCl$_2$, 10% glycerol; pH 6.4; filter sterilized). The cells were incubated on ice for 20 min and then centrifuged again to collect pellet as described previously. The pellet was resuspended in 10 mL ice cold CCMB80 buffer. Chilled CCMB80 was added to the resuspension until OD$_{600}$ of a mixture of 200 µL SOC (0.5% (w/v) yeast extract 2% (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 20 mM MgSO$_4$, 20mM glucose) and 50 µL of resuspended cells was 1.0-1.5. Finally, the cell suspension was aliquotted to 1.5 mL prechilled microcentrifuge tubes and stored at -80°C.

Transformation of chemically competent cells was performed by standard protocol. 50 µL of competent cells are mixed with 1 µL of plasmid solution (50 µg/mL) or 5 µL of a DNA ligation reaction and incubated for 30 min on ice. The mixture was subjected to a 42 °C heat shock for one min and immediately placed back onto ice for 2 min. 400 µL of SOC medium was added to the mix and incubated for one h at 37 °C. Different volumes of mix was plated into LB agar plate with ampicillin and incubated overnight at 37 °C.

II.10 Transformation of *M. acetivorans*

Polyethylene glycol (PEG) mediated transformation was performed as described by Oelgeschlager *et al.* (2009) with some modifications. *M. acetivorans* cells were grown to mid log phase (OD$_{600}$ 0.5 to 0.8) in HSMA medium. The 10 mL culture was centrifuged and the pellet was resuspended in 5 mL of transformation buffer (TB) (0.05 M Pipes sodium salt, 0.35 M D(+) sucrose, 0.6 M NaCl, 1 mM MgCl$_2$, 0.0001 % resazurin, 0.05 % cysteine-HCl, 1 mM dithiothreitol; pH adjusted to 6.8). The cells were centrifuged again and resuspended in 500 µL TB. Three µg of DNA in nuclease free water was either collected on the same day, or taken into the glove bag, or it was kept in the glove bag for up to one week before use. The DNA was mixed with TB containing 40% PEG 4000. 500 µl of prepared cells was mixed well with DNA-PEG complex solution and incubated at room temperature for 4 hours in an anaerobic glove bag. The
entire volume of DNA-PEG complex solution was transferred to a culture tube containing 10 mL of HSMA medium and incubated overnight (12-16 hours) at 37 °C. The next day, the culture was centrifuged at 7000 rpm for 10 min and pellet was resuspended in 500 µL of TB buffer. Appropriate volumes were plated into agar plates with puromycin (2µg/ml) as a selectable marker and incubated in an anaerobic jar inside the glove bag with a headspace of 79.9% N₂, 20% CO₂ and 0.1% H₂S gas mix for 12-14 d.

II.11 Markerless genetic exchange method

pTL1, pTG1, pTO1, pTS1 were transformed (section II.10) into *M. acetivorans* WWM73 and plated onto HSMA agar plates with puromycin (2µg/mL) as a selection marker in an anaerobic jar with N₂/CO₂/ H₂S (79.9%/20%/0.1%) gas. After approximately 2 weeks, individual colonies were picked and inoculated into liquid HSMA media without any selection pressure to resolve the merodiploid state. Afterward, each culture was plated onto an HSMA agar plate containing 8-ADP with 10⁻³-10⁻⁵ dilutions. After 2 weeks, individual colonies were picked and inoculated into liquid HSMA with 8-ADP. When the culture reaches mid-log phase, each culture was plated as described before. Individual colonies were inoculated into liquid HSMA plate with 8-ADP again (Pritchett *et al*. 2004). When the cultures reached stationary phase, PCR screening using primer P1 and P4 of each gene was carried out. PCR screening positive cultures was confirmed by Southern blot as described in section II.22.

II.12 Preparation of genomic DNA from *M. acetivorans*

The culture was grown to stationary phase and centrifuged at 5,624 x g to collect the cell pellet. The pellet was resuspended in TE/NaCl buffer solution. SDS was added to 1% final concentration and mixed gently. Five µl of 2 mg/mL RNase (RNase A/T1 Mix, Thermo Scientific Inc.) was added and mixed well. The mixture was incubated at 37 °C for 25 min. 800 µl of Tris-saturated phenol adjusted to pH 7.9 was added and centrifuged at 16,873 x g for 10 min. The aqueous (upper) layer was transferred to a new tube and mixed with 1 mL of phenol: chloroform: isoamyl alcohol (25:24:1). The mixture was centrifuged at 16,873 x g and the aqueous layer was again transferred to a
new tube and mixed with 1 mL of chloroform, centrifuged at 16,873 x g and aqueous layer was collected in new tube. Ice-cold 100% ethanol (double the volume of aqueous layer collected in the tube), NaCl (0.2 M final concentration in the solution) from 5 M stock was added and placed at -20 °C for 25 min. The mixture was centrifuged as above for 25 min and the supernatant was discarded. The pellet was washed with 70% ethanol twice and resuspended in 100 µl of TE buffer and kept at 37 °C for 10 min to dissolve the DNA pellet in TE buffer.

**II.13 Preparation of plasmid DNA from *E. coli* and PCR product purification**

Plasmid DNA was prepared from transformed *E. coli* EC100 using Wizard® Plus SV Minipreps DNA Purification System while purification of PCR products was performed using Wizard® SV Gel and PCR Clean-Up System from Promega according to manufacturer’s instructions.

**II.14 DNA quantification**

DNA concentration was determined by measuring the absorbance at 260 nm. For DNA, OD\textsubscript{260} equal to 1 corresponds to a 50 µg/ mL concentration. OD\textsubscript{260}/OD\textsubscript{280} ratios of 1.8-2.0 were considered sufficiently pure DNA for use.

**II.15 Electrophoresis of DNA**

Agarose gels (0.8-1% (w/v) in TAE-buffer (Tris-acetate (pH 8.0) 40 mM, EDTA 1mM)) was prepared for separation and analysis of DNA-fragments. 0.5 µL of ethidium bromide (stock 60 µg/µL) was added to 60 ml of agarose gel just prior to casting to make a final concentration of ethidium bromide at 0.5µg/ml. DNA samples were mixed with Blue/Orange Loading Dye from Promega to visualize the progress of DNA separation. 1Kb DNA Ladder mix (Promega or NEB) was used as a standard according to manufacturer’s instructions.

**II.16 Polymerase chain reaction (PCR) for amplifying DNA Fragments**

A pair of oligonucleotide primers were designed for each DNA fragment amplification. Restriction sites were introduced in the primers, as required. All primers
were purchased from Integrated DNA Technology (IDT) Inc. In a typical PCR reaction, 50 ng of genomic DNA or 10 ng of plasmid DNA was used as template. Phusion® High-Fidelity DNA Polymerase (New England BioLabs (NEB)) was used for all cloning experiments whereas VentR® DNA Polymerase (NEB) was used for other screening purposes where high fidelity was not required. Typically, 5 µl of M. acetivorans liquid culture was used as template with VentR® DNA Polymerase while amplifying any gene from whole cell. PCR mix for both polymerases were prepared according to manufacturer’s instruction. All the primers used in this work are listed in Table 2.
Table 2. List of Primers used in this work. Nucleotide sequences comprising restriction endonuclease restriction sites are printed in lower case italics, the priming regions of the primers are printed in capital letters.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Application</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtmP-P1</td>
<td>Generation of gene fragment Upstream of <em>mtmP</em></td>
<td>gcgccacgctgCTCCTGATTAACCC GGAATTC</td>
</tr>
<tr>
<td>mtmP-P2</td>
<td></td>
<td>tggatatatctctggccttggctgatagGGC ATTGGAGACGAATGACA</td>
</tr>
<tr>
<td>mtmP-P3</td>
<td>Generation of gene fragment Downstream of <em>mtmP</em></td>
<td>tccattgcctgtcctcaatgctgaCT ATCAGCCCAAAGGCCAG</td>
</tr>
<tr>
<td>mtmP-P4</td>
<td></td>
<td>gcgcgcagcetGTCGGCGGGAAACCTCC</td>
</tr>
<tr>
<td>mtbP-P1</td>
<td>Generation of gene fragment Upstream upstream of <em>mtbP</em></td>
<td>gcgccacgctgCGACACAATGGAAC TCGGAC</td>
</tr>
<tr>
<td>mtbP-P2</td>
<td></td>
<td>ggggatgtcagagtggcattcgcattgctaG TGTGTCGTCCATAGATTC</td>
</tr>
<tr>
<td>mtbP-P3</td>
<td>Generation of gene fragment Downstream of <em>mtbP</em></td>
<td>taaaatgggtatacatatgagcagacaaatGG CAATGGCAACTGCCATCC</td>
</tr>
<tr>
<td>mtbP-P4</td>
<td></td>
<td>gcgcgcagcetGATCTCAACATGG CTTCGG</td>
</tr>
<tr>
<td>mttP1-P1</td>
<td>Generation of gene fragment upstream of <em>mttP1</em></td>
<td>gcgccacgctgAGGTGAACAGTTCG AACAGG</td>
</tr>
<tr>
<td>mttP1-P2</td>
<td></td>
<td>accaataacagacagacgctgcaagatG CCTCCATTTCGCCATGC</td>
</tr>
<tr>
<td>mttP1-P3</td>
<td>Generation of gene fragment Downstream of <em>mttP1</em></td>
<td>gattcctagcctgaaaaatggaggtaa CTGTGCGGAGCCTC</td>
</tr>
<tr>
<td>mttP1-P4</td>
<td></td>
<td>gcgcgcagcetATCATGTTCCTGCTG CAGAC</td>
</tr>
<tr>
<td>mttP2-P1</td>
<td>Generation of gene fragment upstream of <em>mttP2</em></td>
<td>gcgcgcacgctgCTGGGCAAGGGCTGA TAAAGC</td>
</tr>
<tr>
<td>mttP2-P2</td>
<td></td>
<td>gcgccatattcctctctgaggtttcgaACG TGACGGCATTAAATGC</td>
</tr>
<tr>
<td>mttP2-P3</td>
<td>Generation of gene fragment downstream of <em>mttP2</em></td>
<td>gcggtgatcagctatagcctgctgatgaaAACCCTCAGAGGAGGATG</td>
</tr>
<tr>
<td>mttP2-P4</td>
<td></td>
<td>gcgcgcagctgTACATATAGTGACC TCCGGGAC</td>
</tr>
<tr>
<td>mtmP probe-F</td>
<td>Generation of probe for Δ<em>mtmP</em> detection</td>
<td>CTGGGCTGACAAAGATCGGCG</td>
</tr>
<tr>
<td>mtmP probeR</td>
<td></td>
<td>CCCGTGAGTATAGCAGGAATAT</td>
</tr>
<tr>
<td>mtbp probe-F</td>
<td>Generation of probe for Δ<em>mtbp</em> detection</td>
<td>CTACTATG GACCATTTCC AGGAC</td>
</tr>
<tr>
<td>mtbp probe-R</td>
<td></td>
<td>CGATGAGGGAGTCAATGGAG</td>
</tr>
<tr>
<td>mttP1 probe-F</td>
<td>Generation of probe for Δ<em>mttP1</em> detection</td>
<td>CTGGGCTGACAAAGATCGGCG</td>
</tr>
<tr>
<td>mttP1 probe-R</td>
<td></td>
<td>CCCGTGAGTATGACGGAATAT GC</td>
</tr>
<tr>
<td>mttP2 probe-F</td>
<td>Generation of probe for Δ<em>mttP2</em> detection</td>
<td>CTCTTATACGTACCTTATGC</td>
</tr>
<tr>
<td>mttP2 probe-R</td>
<td></td>
<td>CCCGTGAGTATGACGGAATAT GC</td>
</tr>
</tbody>
</table>
II.17 Enzymatic Digestion of DNA
Restriction endonucleases (NEB) were used for digestion of double stranded DNA. Digestion reactions with buffers, enzyme, and DNA were prepared according to manufacturer’s instructions. Restriction digestion was carried out for 3 h at 37 °C. Gel electrophoresis was performed on the digested sample and the DNA fragment of interest was excised from the gel and purified (section II.13).

II.18 Ligation of DNA fragments
The ratio of insert to vector was added in 5:1 to 10:1 molar concentration. 100 ng of plasmid DNA was used in the ligation reactions. Ligations were carried out in 20 µL reaction mixtures using T4 DNA ligase (NEB) according to manufacturer’s instructions. Ligation reactions were incubated overnight at 16 °C.

II.19 Preparation of fused upstream and downstream DNA fragments of targeted genes by overlap extension PCR and cloning into pMP44
Upstream fragments and downstream fragments were amplified using primer combinations P1/P2 and P3/P4 for each gene in separate PCR reactions, as described in Kohler and Metcalf (2012). The correct sized band was excised and cleaned up from agarose gel (section II.13) and quantified using by absorbance at 260 nm. Another PCR reaction was performed with equimolar amounts of upstream and downstream fragment using forward primer of upstream fragment (P1) and reverse primer of downstream fragment (P4) of the respective genes. The PCR product was subjected to electrophoresis in an agarose gel and the correct sized fragment was excised and purified (Section II.13). The fused upstream and downstream fragments of mtmP, mtbP and mttP1 were digested with PmlI and SacI whereas mttP2 was digested with KpnI and PmlII. The pMP44 was digested with the corresponding restriction enzymes and the digested insert was ligated into the digested vector as described in section II.18.

II.20 Plasmid DNA sequencing
The presence of the correct sequence in recombinant plasmids was determined by the Sanger dideoxy DNA sequencing method (Sanger et al., 1977). Analysis of the
sequencing results was performed using Sequencher 5.1 software (Gene Code Corporation) available at Miami University’s CBFG facility.

II.21 Preparation and labelling of probes for hybridization

Primer sets, mtmP probe-F/mtmP probe-R, mtbP probe-F/mtbP probe-R, mttP1 probe-F/mttP1 probe-R, and mttP2 probe-F/mttP2 probe-R, were used to generate probes for ΔmtmP, ΔmtbP, ΔmttP1, and ΔmttP2 detection, respectively. The sizes of probes were 230 bp for the mtmP probe, 253 bp for the mtbP probe, 218 bp for the mttP1 probe and 288 bp for the mttP2 probe. Biotin labeling of the generated probe was carried out using the Label IT® µArray® Biotin Labeling Kits (Mirus) according to the manufacturer’s instructions.

II.22 Southern blot analysis

Three µg of genomic DNA was digested with EcoRI for ΔmtmP detection, NcoI for ΔmtbP or ΔmttP1 detection, and PstI for ΔmttP2 detection. The digested samples were run in 0.8% agarose gels containing ethidium bromide (0.5µg/ml) and visualized by UV detection along with a scale marker to measure the bands and a size marker ladder. The image captured with scale and ladder was used to analyze band size in the blot. Digested genomic DNA was transferred from the agarose gel onto nylon membrane (NEN™ Life Science Products) using the standard protocol described in Sambrook et al. (1989). Detection of probe hybridization was performed using Biotin Chromogenic Detection Kit (Thermo Scientific) according to the manufacturer’s instructions. The band size for each sample was compared with the digested gel image along with scale to determine whether it is wild type or a knockout mutant. Predicted parental band and knockout band sizes for each gene are listed in the Table 3.
Table 3. List of gene knock outs and the restriction enzymes (RE) used in Southern analysis to check for the predicted DNA fragment sizes in parental and knockout strains.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>RE used for digesting genomic DNA</th>
<th>Fragment Size in Parental strain</th>
<th>Fragment size in knockout strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtmP</td>
<td>EcoRI</td>
<td>~5000 bp</td>
<td>~3500 bp</td>
</tr>
<tr>
<td>mtbP</td>
<td>NcoI</td>
<td>~2000 bp</td>
<td>~800 bp</td>
</tr>
<tr>
<td>mtiP1</td>
<td>NcoI</td>
<td>~2800 bp</td>
<td>~1800 bp</td>
</tr>
<tr>
<td>mtiP2</td>
<td>PstI</td>
<td>~2200 bp</td>
<td>~1500 bp</td>
</tr>
</tbody>
</table>
III. RESULTS

III.1 Bioinformatic analysis shows putative methylamine permeases belong to the APC superfamily of transporters

The transmembrane helices of each of the methylamine permeases were analysed using the TMHMM Server v. 2.0 provided by the Centre for Biological Sequence Analysis (CBS), Technical University of Denmark DTU (http://www.cbs.dtu.dk/services/TMHMM). The characteristic APC fold of 5 transmembrane folds, with additional 1-3 transmembrane segments (TMS) at the C- or N-terminus (Wong et al., 2012), have been predicted for the putative MMA permease (MtmP; 51,100 Da), DMA permease (MtbP; 54,702 Da) and TMA permease (MttP1; 38,207 Da) (Figure 4). MtmP, MtbP, and MttP1 have been predicted to have 12, 11 and 10 TMS respectively (Figure 4A, 4B, 4C). However, another predicted TMA permease, MttP2 (30,197 Da), has been predicted to have 9 transmembrane segments (Figure 4D). MttP1 and MttP2 have high sequence conservation with 86% amino acid sequence identity. MttP2 is truncated and lacks an N-terminal portion that MttP1 contains. An open reading frame (Orf; MA_0930) located alongside mttP2 (Figure 3) encodes a small protein (8,285 Da) with very high sequence identity to the N-terminal portion of MttP1. It seems that one of the TMA permease proteins may be composed of two proteins, MttP2 and MA_0930, which complement each other to become a functional APC superfamily permease with the characteristic five transmembrane folds.

III.2 MtmP and MtbP belong to the Amino Acid-Polyamine-Organocation (APC) Family whereas MttP1 and MttP2 belong to the Betaine/Carnitine/Choline Transporter (BCCT) Family of the APC superfamily.

The protein BLAST program (http://www.uniprot.org/blast/) was used to identify related proteins with >45% identity for each permease. Cladograms for MtmP, MtbP, MttP1 and MttP2 were constructed using the Clustal Omega Multiple Sequence Alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Figures 5, 6 and 7). A cladogram shows *M. acetivorans* mtmP being closely related to predicted methylamine permeases of other *Methanosarcina* and *Methanolobulus* species and to general amino
acid permeases of different methanogenic species (Figure 5). All of the proteins in the cladogram belong to the APC family of transport proteins suggesting MtmP belongs to the same group of proteins. Similarly, the cladogram including *M. acetivorans* MtbP also shows that MtbP is closely related to predicted methylamine permeases of other *Methanosarcina* species and to general amino acid permeases of other methanogenic species (Figure 6). All of the proteins in the cladogram belong to the APC family of transporter proteins suggesting MtbP belongs to same group of proteins. The cladogram with predicted TMA permeases of *M. acetivorans* shows that both MttP1 and MttP2 are closely related to predicted TMA permeases and general amino acid permeases from various methanogenic species (Figure 7).

**III.3 Construction of the *M. acetivorans* DJF17 (*Amtp*) mutant (performed by Tomislav Ticak)**

A 1441 bp fragment of chromosomal DNA containing the *mtmP* gene was deleted from *M. acetivorans* WWM73 using a markerless genetic exchange method and the selected colonies were screened by PCR amplification (Figure 8A), as described in Section II.11. The PCR amplification of an approximate 3300 bp DNA fragment, observed in Figure 8A lanes 7, 8, 9, 10, 12, 13 and 15, is consistent with deletion of the targeted 1441 bp *mtmP* fragment (i.e., positive screening result), whereas the approximate 4800 bp DNA fragment observed in lanes 2, 4, 6, 11 and 14 is the expected size for the non-deleted, wild type *mtmP* fragment (i.e., negative screening result). Genomic DNA from suspected *mtmP* deletion (Figure 8A, lanes 7, 8, 9 10) and wild-type strains was analyzed by Southern hybridization with a *mtmP* probe complementary to a DNA sequence 230 bp upstream of *mtmP*. An *EcoRI* fragment of approximately 5000 bp was detected in wild-type DNA (Figure 8B, lane 1) whereas an approximate 3500 bp fragment was detected in DNA from colonies screened by PCR amplification and suspected to contain the targeted 1441 bp deletion of chromosomal DNA (Figure 8B, lanes 2, 3, and 4). However, a mixture of both wildtype and knockout was detected in one sample (Figure 8B, lane 3) that screened as positive by PCR amplification (Figure 8A, lane 10) The size of the wild-type band (~5000 bp) and knockout bands (~3500 bp) were determined by comparing the Southern hybridization bands (Figure 8B) with the
ethidium bromide stained agarose gel image that contained a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data not shown). The 30 mm on the scale ruler is equal to approximately 5000 bp and 35 mm is approximately 3500 bp.

**III.4 The *M. acetivorans* DJF17 (ΔmtmP) shows an extended lag during MMA-dependent growth**

A direct comparison of growth curves for *M. acetivorans* WWM73 and the ΔmtmP deletion strain (DJF17), described above, in 40mM MMA as sole carbon source was performed. The growth curve revealed that ΔmtmP shows an extended lag phase compared to WWM73. The ΔmtmP strain entered log phase only after 120 h of inoculation, however, by 156 h of incubation both strains reached comparable OD₆₀₀ in their stationary phase (Figure 9A). A growth comparison of ΔmtmP and WWM73 was also performed at low substrate concentration (1 mM MMA) to mimic the physiological conditions where substrate availability is much lower than used under typical laboratory conditions. The growth curves in 1mM MMA again showed an extended lag phase for ΔmtmP when compared with WWM73 (Figure 9B). As a control, growth curves of WWM73 and DJF17 (ΔmtmP) showed no effect of the ΔmtmP deletion on growth with 125mM methanol/40mM acetate (Figure 9C).

**III.5 Construction of the *M. acetivorans* DJF22 (ΔmtbP) mutant**

A 1288 bp DNA fragment containing the majority of the mtbP gene was deleted from the *M. acetivorans* WWM73 chromosome using a markerless genetic exchange method and the selected colonies were screened for deletion by PCR amplification (Figure 10A), as described in Section II.11. PCR amplification of the approximate 2200 bp DNA fragment observed in Figure 10A, lanes 1, 2 and 3 suggests deletion of the targeted 1288 bp mtbP fragment (i.e., positive screening result) whereas the approximate 3600 bp DNA fragment observed in lanes 4 and 5 is the expected size for the non-deleted, wild type mtbP fragment (i.e., negative screening result). Genomic DNA from the suspected strains was analyzed by Southern hybridization with a DNA probe complementary to a 253 bp sequence downstream of mtbP. A *Nco*I-digested DNA
fragment of approximately 2000 bp was detected in WWM73 whereas an approximate 800 base pair fragment was detected in the colonies that screened positive by PCR amplification, consistent with deletion of the targeted 1288 bp fragment of chromosomal DNA (Figure 10B). The sizes of wild-type (~2000 bp) and knockout (~800 bp) bands were determined by comparing the Southern hybridization bands (Figure 10B) with the ethidium bromide stained agarose gel image that contained a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data not shown). The 2.5 cm mark on the scale ruler is approximately 2000 bp and 3.7 cm mark is approximately 800 bp (Figure 10B).

III.6 Construction of *M. acetivorans* DJF37 (ΔmttP1/ΔmttP2) mutants

For construction of the ΔmttP1/ΔmttP2 (DJF37) double deletion strain, the ΔmttP2 strain (DJF32) was prepared first and then mttP1 was deleted from the ΔmttP2 strain. A 798 bp DNA fragment containing the mttP2 gene was first deleted from the *M. acetivorans* WWM73 chromosome using the markerless genetic exchange method (Section II.11) and the selected colonies were screened by PCR amplification. The PCR amplification of ~2000 bp DNA fragment observed in Figure 11A lanes 3, 6 and 7 is a positive screening for deletion of the targeted 798 bp fragment whereas the DNA fragment of ~2800 bp observed in lanes 1, 2, 4, 5, 8, 9, and 10 is the expected size for the non-deleted, wild type mttP2 fragment (i.e., negative screening result). Genomic DNA from suspected deletion strains was analyzed by Southern hybridizations with a DNA probe complementary to a 288 bp DNA sequence upstream of mttP2. A *PstI*-digested DNA fragment of approximately 2200 bp was detected in WWM73 whereas an approximate 1500 bp fragment was detected in cultures that screened positive by PCR amplification, consistent with chromosomal deletion of the targeted 798 bp fragment (Figure 11B). The sizes of wild-type (~2200 bp) and knockout (~1500 bp) bands were determined by comparing the Southern hybridization bands (Figure 11B) with the ethidium bromide stained agarose gel image that contained a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data not shown). The 29 mm mark on the ruler scale is approximately 2200 bp and 34 mm mark is approximately 1500 bp (Figure 11B).
Using a similar strategy, a 968 bp fragment of the mttP1 gene was deleted from the *M. acetivorans ΔmttP2* strain (DJF32 above) and the selected colonies were screened by PCR amplification to detect the *mttP1* deletion. Genomic DNA from suspected double deletion strains was analyzed by Southern hybridization with a *mttP1* DNA probe complementary to a 218 bp DNA sequence upstream of *mttP1*. A *NcoI*-digested DNA fragment of approximately 2800 bp was detected in WWM73 whereas an approximate 1800 bp DNA fragment was detected in the colonies that screened positive by PCR amplification, suggesting deletion of the targeted 968 bp chromosomal fragment (Figure 12A). Sizes of the wild-type (~2800 bp) and knockout (~1800 bp) bands were determined by comparing the Southern hybridization bands (Figure 12A) with the ethidium bromide stained agarose gel image that contained a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data not shown). The 23 mm mark in the ruler scale is approximately 2800 bp and 28 mm mark is approximately 1800 bp (Figure 12A).

As a precaution, Southern hybridization with a probe complementary to a 288 bp DNA sequence upstream of *mttP2* was performed on *NcoI*-digested genomic DNA from the strain constructed above to confirm that the ΔmttP1/ΔmttP2 double deletion strain taken to be DJF37 still contained the mttP2 deletion (Figure 12B). The size of the wild-type (~2200 bp) and knockout (~1500 bp) bands were determined by comparing the Southern hybridization bands (Figure 12B) with the ethidium bromide stained agarose gel image that contained a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data not shown). The 26 mm mark on the scale is approximately 2200 bp and 33 mm mark is approximately 1500 bp (Figure 12B). Together, the above data consistent with DJF37 containing both mttP1 and mttP2 deletions.

### III.7 Construction of the *M. acetivorans DJF27 (ΔmttP1)* mutant

A single *mttP1* gene knockout, resulting in DJF27, from *M. acetivorans* WWM73 was also prepared as described above for preparation of the *mttP1/mttP2* double deletion mutant. In brief, a 968 bp fragment of the *mttP1* gene was deleted from *M. acetivorans* WWM73 and the selected colonies were screened by PCR amplification.
The PCR amplification of an approximate 2000 bp DNA fragment (i.e., positive screening), as observed in Figure 13A lanes 2, 3, and 5 is consistent with deletion of the targeted 968 bp fragment whereas the ~3000 bp DNA fragment observed in lanes 4 and 6 is a negative screening, indicating that the targeted 968 bp is included in the amplified fragment (Figure 13A). Genomic DNA from suspected mttP1 deletion strains was analyzed by Southern hybridization with a mttP1 DNA probe complementary to a 218 bp DNA sequence upstream of mttP1. A NcoI-digested DNA fragment of approximately 2800 bp was detected in WWM73 (Figure 13B, lane 1) whereas an approximate 1800 bp DNA fragment was detected in the colonies that screened positive by PCR amplification, suggesting deletion of the targeted 968 bp chromosomal fragment (Figure 13B, lanes 2, 4). However, a mixture of both wild-type and knockout was detected in one sample (Figure 13B, lane 3) that screened as positive by PCR amplification (Figure 13A, lane 3). The size of the wild-type (~2800 bp) and knockout (~1800 bp) bands were determined by comparing the Southern hybridization bands (Figure 13B) with the ethidium bromide stained agarose gel image that contained a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data not shown). The 24 mm mark in the ruler scale is approximately 2800 bp and 29 mm is approximately 1800 bp (Figure 13B).

III.8 The M. acetivorans DJF22 (ΔmtbP) and DJF37 (ΔmttP1/ΔmttP2) show no clear defect on growth with 40mM DMA or 20 mM TMA

A direct comparison of growth between M. acetivorans WWM73 and the ΔmtbP deletion strain (DJF22) in 40 mM DMA was performed (Figure 14A). Although the overall growth curves of WWM73 and ΔmtbP were similar, the ΔmtbP strain showed a lag and grew slightly slower than WWM73, reaching stationary phase at OD₆₀₀ ~0.4 whereas WWM73 grew to ~0.5 in the same amount of time. Growth of ΔmtbP and WWM73 was also compared at low substrate concentration (1mM DMA) to mimic the physiological conditions where substrate availability is much lower than used under typical laboratory conditions. The growth comparison of ΔmtbP and WWM73 in 1mM DMA showed a significant growth defect by the ΔmtbP strain (Figure 14B). As a
control, growth curves of WWM73 and ΔmtbP showed no effect of the ΔmtbP deletion on growth with 125mM methanol/40mM acetate (Figure 14C).

Growth curves of *M. acetivorans* WWM73 and DJF37 (ΔmttP1/ΔmttP2) did not reveal a growth defect by the ΔmttP1/ΔmttP2 mutant in 20 mM TMA (Figure 15A). WWM73 and DJF37 displayed almost overlapping growth curves, indicating that deletion of *mttP1* and *mttP2* does not affect TMA-dependent growth under the conditions examined. Although growth of WWM73 and DJF37 at low substrate concentration (1mM TMA) showed some similarity, DJF37 (ΔmttP1/ΔmttP2) showed a higher growth yield than WWM73 (Figure 15B). As a control, growth curves of WWM73 and DJF37 (ΔmttP1/ΔmttP2) showed no effect of the double deletion on growth with 125mM methanol/40mM acetate (Figure 15C).

**III.9 Construction of the *M. acetivorans* DJF52 (ΔmtmP/ΔmtbP) mutant**

The DJF52 double mutant (ΔmtmP/ΔmtbP) was prepared by deleting a 1288 bp DNA fragment, containing the majority of the *mtbP* gene, from strain DJF17 (ΔmtmP) using a markerless genetic exchange method. Selected colonies were screened by PCR amplification for deletion of *mtbP* from DJF17 (Figure 16A), as described in Section II.11. The PCR amplification of an approximate 2200 bp DNA fragment observed in Figure 16A, lanes 2 and 3 is a positive screening, suggesting deletion of the targeted 1288 bp fragment, whereas the approximate 3600 bp DNA fragment observed in lane 4 is a negative screening, indicating that the targeted 1288 bp is included in the amplified fragment and was not deleted from the chromosome. Genomic DNA from the suspected deletion strains was analyzed by Southern hybridization with a DNA probe complementary to a 253 bp sequence downstream of *mtbP*. A *NcoI*-digested DNA fragment of approximately 2000 bp was detected in WWM73 whereas an approximate 800 bp fragment was detected in DNA from colonies that screened positive by PCR amplification, consistent with deletion of the targeted 1288 bp fragment of chromosomal DNA (Figure 16B). The sizes of wild-type (~2000 bp) and knockout (~800 bp) bands were determined by comparing the Southern hybridization bands (Figure 16B) with the ethidium bromide stained agarose gel image that contained a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data
not shown). The 2.2 cm mark in the ruler scale is approximately 2000 bp and 3.4 cm is approximately 800 bp (Figure 16B).

**III.10 The *M. acetivorans* DJF52 (ΔmmtP/ΔmtbP) shows a growth defect with 20mM DMA**

Growth curves for DJF52 (ΔmmtP/ΔmtbP) and WWM73 were compared with different carbon sources. Growth with 40 mM MMA revealed a longer lag phase for DJF52 in comparison to WWM73 (Figure 17A). Growth in 20mM DMA showed a DJF52 growth defect in that stationary phase occurred at ~ 0.2 OD\textsubscript{600} whereas WWM73 continued growing out to ~ 0.5 OD\textsubscript{600} (Figure 17B).

Growth with 20mM TMA indicated DJF52 entered logarithmic growth faster than WWM73 but both eventually reached a similar stationary phase OD\textsubscript{600} of ~ 0.7 (Figure 17C). Growth with 125 mM methanol/40 mM acetate, as a control, was similar for DJF52 and WWM73 but DJF52 may have entered logarithmic growth slightly faster (Figure 17D).

**III.11 Construction of *M. acetivorans* DJF51 (ΔmtbP/ΔmttP1/ΔmttP2) mutant**

The DJF51 triple mutant (ΔmtbP/ΔmttP1/ΔmttP2) was prepared by deleting a 1288 bp DNA fragment, containing the majority of the *mtbP* gene, from strain DJF37 (ΔmttP1/ΔmttP2) and DJF17 (ΔmmtP), respectively, using a markerless genetic exchange method. Selected colonies were screened by PCR amplification for deletion of *mtbP* from DJF37 (Figure 18A), as described in Section II.11. PCR amplification of the ~2200 bp DNA fragment observed in Figure 18A lanes 1, 2, 3, 4 and 5 is a positive screening, suggesting deletion of targeted 1288 bp fragment. Genomic DNA from the suspected deletion strains was analyzed by Southern hybridization with a DNA probe complementary to a 253 bp sequence downstream of *mtbP* (Figure 18B). A *NcoI*-digested DNA fragment of approximately 2000 bp was detected in WWM73 whereas an approximate 800 bp fragment was detected in the colonies that screened positive by PCR amplification, consistent with deletion of the targeted 1288 bp chromosomal fragment (Figure 18B). The size of wild-type (~2000 bp) and knockout (~800 bp) bands were determined by comparing the Southern hybridization bands (Figure 18B) with the
ethidium bromide stained agarose gel image that contained a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data not shown). The 1.8 cm mark in the ruler scale is approximately 2000 bp and 3.0 cm is approximately 800 bp.

III.12 The *M. acetivorans* DJF51 (ΔmtbP/ΔmttP1/ΔmttP2) shows a growth defect with 20mM DMA

Growth of DJF51 and WWM73 with different carbon sources was measured and compared. Growth of DJF51 and WWM73 in 40mM MMA showed almost overlapping growth curves (Figure 19A) whereas growth in 20 mM DMA reached stationary phase at different times and culture densities. DJF51 (ΔmtbP/ΔmttP1/ΔmttP2) reached stationary phase at OD$_{600}$ ~0.24 whereas WWM73 continues growing until at least OD$_{600}$ ~0.5 (Figure 19B).

The growth curves of DJF51 and WWM73 in 20 mM TMA reveal a slower growth rate for DJF51 as compared to WWM73, however, both strains showed comparable stationary phase levels of growth (Figure 19C). In addition, growth curves of DJF51 and WWM73 revealed that DJF51 entered logarithmic growth significantly faster than WWM73 on 125 mM methanol/40 mM acetate (Figure 19D).
Figure 4. Predicted transmembrane helices of putative methylamine permeases was determined using the TMHMM Server v 2.0 provided by Centre for Biological Sequence Analysis (CBS) Technical University of Denmark DTU (http://www.cbs.dtu.dk/services/TMHMM). A. MtmP, B. MtbP, C. MttP1, and D. MttP2 were predicted to contain 12, 11, 10 and 9 transmembrane helices, respectively. The y-axis shows the probability of the polypeptide being in the transmembrane region (red), inside the cytoplasm (blue) or on the exterior of the cell (pink) while the x-axis represents the amino acids in the polypeptide chain.
Figure 5. Cladogram of the putative MMA permease and related proteins. The cladogram was constructed using the Clustal Omega Multiple Sequence Alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/) using 10 proteins, with >45% identity to the putative MMA permease, generated by a protein BLAST program (http://www.uniprot.org/blast/). The numbers at each branch denote the terminal branch length (i.e., distance to the last node). Uniprot accession numbers for each protein are listed in front of their protein names.
Figure 6. Cladogram of the putative DMA permease and related proteins. The cladogram was constructed using the Clustal Omega Multiple Sequence Alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/) using 10 proteins, with >45% identity to the putative MMA permease, generated by a protein BLAST program (http://www.uniprot.org/blast/). The numbers at each branch denote the terminal branch length (i.e., distance to the last node). Uniprot accession numbers for each protein are listed in front of their protein names.
Figure 7. Cladogram of the two putative TMA permeases and related proteins. The cladogram was constructed using the Clustal Omega Multiple Sequence Alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/) using 10 proteins, with >45% identity to the putative MMA permease, generated by a protein BLAST program (http://www.uniprot.org/blast/). The numbers at each branch denote the terminal branch length (i.e., distance to the last node). Uniprot accession numbers for each protein are listed in front of their protein names.
Figure 8. Screening and confirmation of the mtmP deletion from WWM73 using the markerless genetic exchange method. A. Fourteen colonies were screened by PCR amplification using the primers-mtmP-P1 and mtmP-P4 (Table 2). Seven out of 14 colonies (lanes 7, 8, 9, 10, 12, 13, and 15) yielded 3 kb products indicating that they screened positive for a deletion (KO). Band sizes of ladder (in bp) is indicated along the left side of the ladder (lane L). B. Confirmation of positively screened colonies (Lanes 7, 8, 9, and 10) was carried out by Southern hybridization. Lanes 2, 3, and 4 contained a deletion of the expected size and are assumed to contain the mtmP knockout while lane 5 showed a mixture of both mtmP knockout and WWM73 hybridization signals. Genomic DNA from WWM73 used in lane 1 as a negative control. The size of wild-type (~5000 bp) and knockout (~3500 bp) bands were estimated by comparing the Southern hybridization bands with an image of the ethidium bromide stained agarose gel used for the Southern blot, with the gel containing a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data not shown).
Figure 9. Growth curves of DJF17 (ΔmtmP) and WWM73 on different substrates. For each graph, the y-axis shows optical density at 600 nm and the x-axis shows time in hours. The growth curves were carried out in triplicate and standard error bars were assigned for each time point. A. Growth curves of ΔmtmP and WWM73 with 40 mM MMA as the sole carbon substrate. B. ΔmtmP and WWM73 grown with 1mM MMA. C. ΔmtmP and WWM73 grown with 125 mM methanol/40 mM acetate.
A. 

B. 

C.
Figure 10. Screening and confirmation of the *mtbP* deletion from WWM73. A. Several colonies were screened by PCR amplification using primers- *mtbP*-P1 and *mtbP*-P4 (Table 2). Three of the colonies (lanes 1, 2, and 3) screened positive as a knockout (KO) deletion. Lanes 4 and 5 were negative for a deletion, indicating a wild type (WT) *mtbP* gene. Band sizes of ladder (in bp) is indicated along the left side of the ladder. B. Confirmation of positively screened colonies (lanes 1, 2, and 3) was carried out by Southern hybridization. Lanes 2, 3, and 4 contained a deletion of the expected size and are assumed to contain the *mtmP* knockout (KO). Genomic DNA from WWM73 (WT) was used in lane 4 as a negative control for the *mtbP* knockout. The size of wild-type band (~2000 bp) and knockout bands (~800 bp) were estimated by comparing the Southern hybridization bands with an image of the ethidium bromide stained agarose gel used for the Southern blot, with the gel containing a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data not shown).
Figure 11. Screening and confirmation of the *mttP2* deletion from WWM73. **A.** Several colonies were screened by PCR amplification using the primers *mttP2*-P1 and *mttP2*-P4 (Table 2). Three out of ten colonies (lanes 3, 6, and 7) were screened positive (KO) and seven (lanes 1, 2, 4, 5, 8, 9, 10) negative (WT) for a deletion. Band sizes of the ladder (in bp) are indicated along the left side of the ladder (lane L). **B.** Confirmation of positively screened colonies (lanes 1, 2, and 3) was carried out by Southern hybridization. Lanes 1, 2, and 3 were contained a deletion of the expected size and are assumed to contain the *mttP2* knockout (KO) while lane 4 contained WWM73 genomic DNA (WT) as a negative control. The size of the wild-type band (~2200 bp) and knockout bands (~1500 bp) were estimated by comparing the Southern hybridization bands with an image of the ethidium bromide stained agarose gel used for the Southern blot, with the gel containing a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data not shown).
Figure 12. Southern hybridizations to confirm deletion of *M. acetivorans* *mttP1/mttP2* genes in DJF37. A. Southern hybridization to confirm deletion of *mttP1* from Δ*mttP2* (DJF32). Three colonies that screened positive by PCR amplification for loss of the *mttP1* fragment (data not shown) were evaluated further by Southern hybridization. All three colonies (lanes 2, 3 and 4) contained a deletion of the expected size and are assumed to contain the *mttP1* deletion. Lane 1 contained WWM73 genomic DNA (WT) as a control. The sizes of the wild-type (WT) (~2800) and knockout (KO) (~1800) bands were estimated by comparing the Southern hybridization bands with an image of the ethidium bromide stained agarose gel used for the Southern blot, with the gel containing a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data not shown). B. Southern hybridization was also used to confirm the *mttP2* deletion in the suspected Δ*mttP1/ΔmttP2* double deletion mutants. Genomic DNA from three colonies suspected to contain the Δ*mttP1/ΔmttP2* deletions were analyzed by Southern hybridization for the *mttP2* deletion. All three colonies (lanes 2, 3 and 4) contained a deletion of the expected size and are assumed to contain the *mttP2* deletion (KO). Lane 1 contained WWM73 genomic DNA (WT) as a control. The sizes of the wild-type (~2200) and knockout (~1500) bands were estimated by comparing the Southern hybridization bands with an image of the ethidium bromide stained agarose gel used for the Southern blot, with the gel containing a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data not shown).
Figure 13. Screening and confirmation of the mttP1 deletion from WWM73. A. Several colonies were screened by PCR amplification using primers- mttP1-P1 and mttP1-P4 (Table 2). Three colonies (lanes 2, 3, 5) were screened positive and two colonies (Lanes 4 and 6) were screened negative for a deletion. Band sizes of ladder (in bp) are indicated along the left side of the ladder (lane 1). B. Confirmation of three positively screened colonies was carried out by Southern hybridizations. Lanes 2 and 4 contained a deletion of the expected size and are assumed to contain the mttP1 knockout (KO) while lane 3 showed mixture of both mttP1 knockout and WWM73 hybridization signals. WWM73 genomic DNA (WT) was used in lane 1 as a negative control. The size of the wild-type band (~2800 bp) and knockout bands (~1800 bp) were estimated by comparing the Southern hybridization bands with an image of the ethidium bromide stained agarose gel used for the Southern blot, with the gel containing a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data not shown).
Figure 14. Growth curves of DJF22 (ΔmtbP) and WWM73 on different substrates. For each graph, the y-axis shows optical density at 600 nm and the x-axis shows time in hours. The growth curves were carried out in triplicate and standard error bars were assigned for each time point. A. Growth curves of ΔmtbP and WWM73 on 40 mM DMA as the sole carbon source. B. ΔmtbP and WWM73 grown with 1mM DMA C. ΔmtbP and WWM73 grown with 125 mM methanol/40 mM acetate.
**Figure 15.** Growth curves of DJF37 ($\Delta mttP1/\Delta mttP2$) and WWM73 on different substrates. For each graph, the y-axis shows optical density at 600 nm and the x-axis shows time in hours. Growth curves were carried out in triplicate and standard error bars were assigned for each time point. **A.** Growth curves of DJF37 ($\Delta mttP1/\Delta mttP2$) and WWM73 with 20 mM TMA as the sole carbon source. **B.** Growth curves of DJF37 ($\Delta mttP1/\Delta mttP2$) and WWM73 with 1mM TMA. **C.** Growth curves of DJF37 ($\Delta mttP1/\Delta mttP2$) and WWM73 grown with 125 mM methanol/40 mM acetate.
**Figure 16.** Screening and confirmation of the *mtbP* deletion from DJF17 (Δ*mtmP*). A. Five colonies were screened by PCR amplification using the primers *mtbP*-P1 and *mtbP*-P4 (Table 2). Two of the colonies (Lanes 2 and 3) screened positive for a deletion. Band sizes of ladder (in bp) are indicated along the left side of the ladder (lane 1). B. Confirmation of positively screened colonies was carried out by Southern hybridization. Lanes 2 and 3 contained a deletion of the expected size and are assumed to contain the *mtbP* knockout (KO) deletion. WWM73 genomic DNA (WT) was used in lane 1 as a negative control for the *mtbP* knockout. The size of wild-type (~2000 bp) and knockout (~800 bp) bands were estimated by comparing the Southern hybridization bands with an image of the ethidium bromide stained agarose gel used for the Southern blot, with the gel containing a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data not shown).
Figure 17. Growth curves of DJF52 (ΔmtmP/ΔmtbP) and WWM73 on different substrates. For each graph, the y-axis shows optical density at 600 nm and the x-axis shows time in hours. Growth curves were carried out in triplicate and standard error bars were assigned for each time point. A. Growth curves of DJF52 and WWM73 on 40 mM MMA as the sole carbon source. B. DJF52 and WWM73 grown on 20 mM DMA. C. DJF52 and WWM73 grown on 20 mM TMA. D. DJF52 and WWM73 grown with 125 mM methanol/40 mM acetate.
**Figure 18.** Screening and confirmation of the \textit{mtbP} deletion from DJF37 (\(\Delta mtP1/\Delta mtP2\)). \textbf{A.} Five colonies were screened by PCR amplification using primers \textit{mtbP}-P1 and \textit{mtbP}-P4 (Table 2). All five colonies (lanes 2, 3, 4, 5, and 6) screened positive for a deletion (KO). Band sizes of ladder (in bp) are indicated along the left side of the ladder (lane 1). \textbf{B.} Confirmation of positively screened colonies was carried out by Southern hybridization. Lanes 2, 3, 4, 5 and 6 contained a deletion of the expected size and are assumed to contain the \textit{mtbP} knockout (KO). WWM73 genomic DNA (WT) was used in lane 1 as a negative control. The size of the wild-type (~2000 bp) and knockout (~800 bp) bands were estimated by comparing the Southern hybridization bands with an image of the ethidium bromide stained agarose gel used for the Southern blot, with the gel containing a DNA ladder for estimation of fragment size and a ruler to measure migration distance during electrophoresis (data not shown).
**Figure 19.** Growth curves of DJF51 (ΔmtbP/ΔmttP1/ΔmttP2) and WWM73 on different substrates. For each graph, the y-axis shows optical density at 600 nm and the x-axis shows time in hours for each reading. Growth curves were carried out in triplicate and standard error bars were assigned for each time point. **A.** Growth curves of DJF51 and WWM73 on 40 mM MMA as the sole carbon substrate. **B.** DJF51 and WWM73 grown on 20 mM DMA. **C.** DJF51 and WWM73 grown on 20 mM TMA. **D.** DJF51 and WWM73 grown with 125 mM methanol/40 mM acetate.
IV. DISCUSSION

Methanogenesis is vital for *M. acetivorans* as it is the only energy-generating pathway it is capable of using. The importance of this pathway is reflected by the number of genes devoted to this pathway. More than 200 genes involved in methanogenesis from methylamines, acetate, and methanol are present in multiple copies (Galagan *et al.*, 2002). Genes involved in methylotrophic methanogenesis are present in multiple copies, including genes encoding methyl transferases and corrinoid proteins involved in utilization of methanol, MMA, DMA, and TMA. The mechanism of cytoplasmic methylotrophic substrate utilization by *M. acetivorans* is well characterized. However, the mechanism of substrate transport into the cell is still unknown. The aim of this thesis was to identify methylamine transporters present in this organism.

Because methylamines are protonated when contained in media with a pH of 6.5-6.8, they cannot diffuse across membranes and require permeases to be transported into the cell. Protonation and deprotonation of the methylamines is determined by the pH of the medium and its pKa value. The pKa value of methylamines range from 9 to 11 which is 2 to 4 units higher than the pH of the growth media. Therefore, most of the methylamines remain protonated, which means that a permease is presumably required to catalyse transport across the membrane.

Genes encoding methyltransferases and their cognate corrinoid proteins for specific substrates are present in operons within the genome of *M. acetivorans*, as shown in Figure 3. Each of the methylamine catabolic operons contains conserved open reading frames. Bioinformatic analysis of these open reading frames showed that these are transmembrane proteins with characteristic properties of APC family transporters and choline uptake transporters of the Lic-B cation transporter family. This class of transporters include solute-solute antiport transporters $S_1$ (out) + $S_2$ (in) ⇌ $S_1$ (in) + $S_2$ (out) (Schweikhard and Ziegler, 2012). The probable mechanism of methylamine transport catalyzed by putative permeases can be generalized as methylamine (out) + H$^+$ (in) ⇌ methylamine (in) + H$^+$ (out).

Both the position and bioinformatic analysis of the operon-located Orfs led us to hypothesize that these Orfs encode methylamine-specific transporters. Recent
advancements in the development of genetic methods in *M. acetivorans* have provided the necessary tools to answer the question posed in this project. A markerless genetic exchange method has been developed for the deletion of genes from specific loci (Pritchett *et al.*, 2004). This method has been used for creating all of the gene deletion mutants studied in this project. One of the advantages of this technique is that it excises the markers from the genome at the end of the gene deletion step, giving it the name “markerless genetic exchange method. This technique allowed the use of same marker repeatedly in the resultant mutants to target another gene in order to create double mutants and so forth. Similarly, the phenotypic study of different mutants and the wild type WWM73 strain has been possible due to the ability of growing these cells as single cells in high salt media as described by Sowers *et al.* (1993).

The growth studies of DJF17 (ΔmtmP) and WWM73 show that the ΔmtmP mutant is able to grow in MMA as a sole carbon source. However, the ΔmtmP mutant displayed a longer lag phase compared to WWM73. The slower growth rate of ΔmtmP indicates that the cells were able to take-up and utilize MMA but not at the optimal rate. The concentration of MMA in the experimental condition in the laboratory is much higher than the physiological concentration. We used 40mM MMA in our laboratory studies whereas its natural habitat concentration is in micro molar range. Comparative growth studies of ΔmtmP and WWM73 in 1mM MMA showed that the growth curve of both ΔmtmP and WWM73 followed a similar pattern as that in higher substrate concentration (Figure 9A, 9B). This observation suggests that *M. acetivorans* is able to take-up MMA even in lower concentrations at the same rate as in higher concentrations. One possibility for MMA transport into the cell might be the presence of non-specific permeases. Ammonium transporters, AmtA, in *Corynebacterium glutamicum* have been shown to transport methylamine even though AmtA has a lower affinity for methylamine in comparison to ammonium (Walter *et al.*, 2008). Three copies of the ammonium transporter are present in *M. acetivorans* which might be responsible for the non-specific permeability of MMA.

The growth curves of the DJF22 (ΔmtbP) and WWM73 in 20mM DMA were comparable. However, the ΔmtbP growth curve was slightly slower (Figure 14). It might
be possible that non-specific permeases allow DMA to enter the cell in the absence of \textit{mtbP}. Non-specific permeability should be reduced if the level of DMA is reduced. To investigate this possibility, growth of the \textit{\textDelta mtbP} mutant and WWM73 was recorded with 1mM DMA as carbon source. Under these conditions, growth of \textit{\textDelta mtbP} with 1mM DMA was almost undetectable indicating that the presence or absence of \textit{\textDelta mtbP} could make a difference at the physiological concentrations of DMA in their natural habitat.

Deletion of both \textit{mttP1} and \textit{mttP2} from WWM73 (DJF37) did not show any growth defect when grown on TMA (Figure 15A). This suggests that the \textit{\textDelta mttP1/\textDelta mttP2} double mutant strain (DJF37) was able to take-up TMA in a manner indistinguishable from the WWM73 parental strain. The \textit{\textDelta mttP1/\textDelta mttP2} strain did not show any growth defect in the lower concentration of 1mM TMA (Figure 15B), which means that there might be other permease proteins that allow TMA to enter the cell in the absence of the putative \textit{mttP1} and \textit{mttP2} TMA permease genes (Figure 16). Another possibility might be the presence low level of nonprotonated TMA present that results in TMA diffusion across the membrane.

A comparison of the chemical structures of the three different methylamines, MMA, DMA, and TMA, shows that each of them differ from each other only in the number of methyl groups (Figure 20). On the other hand, the putative methylamine permeases MtmP, MtbP, and MttP1 have been predicted to have common mechanisms of transporting their specific methylamine permeases with their characteristic 10 transmembrane helices (Wang et al., 2012) (Figure 4). MttP2 is the exception with 9 transmembrane helices, however, a short Orf upstream of \textit{mttP2} is highly similar to the N-terminal portion of the other TMA \textit{mttP1} permease gene, suggesting that \textit{mttP2} may be split into two domains which probably interact with each other to make a functional MttP2 with the characteristic 10 transmembrane helices. Therefore, the similarity of different methylamine structures and permeases, with probable common transport mechanisms across the membrane, indicate a higher probability of non-specific permeability of these substrates.

The 4-aminobutyric acid (GABA) permease (GabP), an APC class permease from \textit{E. coli}, has been shown to catalyze transport of structurally diverse compounds
like 3-piperidinecarboxylic acid (nipecotic acid), 3-aminopropanoic acid, 5-
aminopentanoic acid, 3-aminobutyric acid and cis-3-aminocyclohexyl carboxylic acid
(Figure 21) (Brechtel et al., 1996a). In addition, a GABA permease from Bacillus
subtilis was shown to complement a GabP mutant in E. coli, which could transport
several GABA analogues (Brechtel et. al., 1996b). This evidence of cross-reactivity of
related substrates in APC class permeases suggests that methylamine permeases (MtM, MtB, MtT1, MtT2) in M. acetivorans might transport two or more types of
methylamines resulting in a lack of complete growth phenotype in the absence of the
putative native permease of the supplied methylamine. The lack of growth inhibition by
the ΔmtmP, ΔmtbP, and ΔmttP1/ΔmttP2 mutants when cultured, respectively, with
MMA, DMA, and TMA as the sole carbon source could be attributed to the lack of
substrate specificity by the different methylamine permeases.

The hypothesis of specific permease(s) for each substrate and, at the same time,
the observation of non-specific permeability of these methylamines seems paradoxical.
It could be explained by the fact that the mutants are grown in much higher substrate
concentration (20-40mM) in pure culture as compared to physiological concentrations (<
1mM). Therefore, a dedicated permease for each substrate might be extremely important
in the natural habitat in order to survive the interspecies and intraspecies competition at
very low substrate availability.

The putative methylamine permease genes reside in close genomic proximity to
their respective substrate-specific methyltransferases and corrinoid protein genes (Figure
3). Methanogenesis is the only source of energy generation in M. acetivorans
(Hedderich and Whitman, 2013), so the process of methanogenesis should be very
efficient in order to be competitive in low substrate habitats. The putative permeases
might directly interact with the substrate-specific methyltransferases and corrinoid
proteins in a complex to directly funnel the substrate from the environment into a
methyltransferase complex for more efficient methanogenesis. Interaction of a permease
with its specific methyltransferase and corrinoid proteins for efficient methanogenesis
might be another reason for maintaining a dedicated permease for each substrate.
In order to check non-specific permeability of different methylamine permeases, mtbP was deleted from ΔmtmP and ΔmttP1/ΔmttP2, thereby generating ΔmtmP/ΔmtbP (DJF52) and ΔmtbP/ΔmttP1/ΔmttP2 (DJF51). Interestingly, both DJF52 (Figure 17) and DJF51 (Figure 19) showed a pronounced growth defect in DMA as the sole carbon source. Growth by DJF51 and DJF52 was attenuated to 0.2 OD_{600} as compared to ~ 0.5 OD_{600} with WWM73. This suggests that MtmP, MttP1, and MttP2 might have a role in non-specific permeability of DMA at 20mM substrate concentration. However, DJF51 (ΔmtbP/ΔmttP1/ΔmttP2) did not show any growth defect in TMA and DJF52 (ΔmtmP/ΔmtbP) show a growth defect in MMA similar to ΔmtmP in MMA. Different permeases likely have different levels of affinity for non-specific substrates. It could be possible that DMA has less affinity for non-specific permeases and therefore started to show some defect in DJF51 and DJF52, whereas, MMA and TMA still have higher affinity for the remaining non-specific permeases in the prepared strain. In order to completely understand the non-specific permeability of these permeases, a quadruple knock out (ΔmtmP/ΔmtbP/ΔmttP1/ΔmttP2) should be prepared and examined on different substrates. It is still possible that the quadruple knockout could grow in one or other substrates because of the presence of the glycine betaine/L-proline ABC transporter. Glycine betaine/L-proline ABC transporter, ProVWX is the Betaine/Carnitine/Choline Transporter (BCCT) family transporter that catalyzes transport of glycine betaine (Barron et al., 1987). Since, glycine betaine is structurally very similar to TMA (Figure 20), the glycine betaine/L-proline ABC transporter present in M. acetivorans might also be involved in non-specific permeability of one or more methylamine substrates. The lack of a growth phenotype by DJF37 (ΔmttP1/ΔmttP2) in TMA as the sole carbon source suggests that an alternative permease may facilitate TMA permeability. The BCCT transporter is a strong candidate for non-specific permeability of one or more methylamines and needs to be addressed in the future. The structure of glycine betaine is similar to TMA because both of them have three methyl groups bonded to a central nitrogen atom. It has been reported that a BCCT family transporter, the glycine betaine symporter BetP present in Corynebacterium glutamicum, has three conserved tryptophan residues that bind to trimethylammonium of glycine betaine. These tryptophan residue are present in the center of the enzyme and form a prism-like cage providing specificity
to substrate binding (Ziegler et. al., 2010). Even though the glycine betaine/L-proline ABC transporter is evolutionarily diverse from BCCT family glycine betaine symporter, the crystal structure of BetP (BCCT family symporter) and ProX (ligand binding subunit of glycine betaine/L-proline ABC transporter) reveal highly similar binding co-ordinates (Ziegler et. al., 2010). Since the active site of the glycine betaine transporter recognizes the trimethylammonium portion of the substrate, it would seem there is high probability that this class of transporter could recognize TMA. The crystal structure of the ligand binding protein (ProX) of glycine/betaine ABC transport protein in *Archaeoglobus fulgidus* has been solved with both glycine betaine and trimethylammonium as a substrate and it shows that both compounds bind at the active binding site with exactly the same orientation (Schiefner et al., 2004a). The crystal structure of glycine/betaine ABC transport protein in *E. coli* has also revealed the same binding properties although these proteins have very low sequence identity to those of *A. fulgidus* (Schiefner et al., 2004b). These findings suggest that two evolutionarily unlinked proteins, the BCCT symporter and glycine/betaine ABC transporter, have a common mechanism of substrate binding and the substrate binding protein (ProX) of the glycine betaine ABC transporter could bind trimethylammonium at its active site. The lack of a growth phenotype by DJF37 (∆mttP1/∆mttP2) on TMA could be due to the presence of the glycine/betaine ABC transport protein (ProVWX). To test this possibility, the gene encoding ProVWX could be knocked out in DJF37 (∆mttP1/∆mttP2) and the growth phenotype in TMA examined. Alternately, expression of proVWX at the mRNA level, or ProVWX at the protein level, in DJF51 (∆mtbP/∆mttP1/∆mttP2) could be observed when grown with methylamine and non-methylamine substrates (e.g., acetate/methanol) and compared to expression in WWM73 when grown with methylamine substrates. In addition, an ammonium transporter has been shown to have a role in monomethylamine transport (Walter et al., 2008). The active site on ammonium transporter and methylammonium are so similar that a change in a conserved histidine within the active pore of the ammonium transporter in *E. coli* altered the conductance of ammonium and methylamines (Wang et al., 2013). The presence of the ammonium transporters might still be able to transport methylamine in the quadruple methylamine permease mutant suggested above. In that case, an alternative approach would be needed to show that
these permeases are necessary. Recently, expression of archaeal membrane proteins in \textit{E. coli} has been shown (Ma et al., 2013). Expression of the different predicted permeases in \textit{E. coli} followed by an uptake assay with labelled substrate could answer our question directly.

\textbf{Concluding remark}

Primary objective of my thesis has been identifying the genes that encode permeases that allow MMA, DMA, and TMA to enter into the cell and take part in methanogenesis pathway. Most of the methanogenic genes that encode substrate specific methyltransferases and corrinoid proteins are well characterized and are cytoplasmic proteins. Lack of knowledge on how these substrates get into the cell has been a missing link in the methanogenic pathway. This project seeks to identify the genes that encode substrate specific permeases. Genomic context suggested the genes to be putative permeases which was further supported by bioinformatic analyses. These analyses provided strong indication that the identified genes, \textit{mtmP}, \textit{mtbP}, \textit{mttP1}, and \textit{mttP2} are putative permease genes for MMA, DMA, TMA, TMA respectively. Three different gene knockout strains, \textit{ΔmtmP}, \textit{ΔmtbP}, \textit{ΔmttP1}/\textit{ΔmttP2} were prepared and their growth phenotypes were studied. The growth phenotypes showed that all of the strains were able to take up their respective substrates in the laboratory condition of pure culture and high substrate concentration. However, the \textit{ΔmtmP} strain showed an extended lag phase as compared to WWM73. It has been reported that GABA permease, an APC class permease, could transport diverse analogues of GABA (Brechtel \textit{et al}., 1996a). In addition, the ligand binding protein (ProX) of glycine/betaine ABC transport protein in \textit{Archeoglobus fulgidus} has been shown to bind both glycine betaine and trimethylammonium as a substrate (Schiefner \textit{et al}., 2004a). The role of an ammonium transporter in MMA permeability have also been reported in \textit{E. coli} (Walter \textit{et al}., 2008).

These findings indicate that there might be non-specific permeability among the methylamine permeases as well as the probability of additional permeases having a role in the permeability of methylamine. To test the possibility of cross-reactivity among methylamine permeases, additional methylamine knockout strains, DJF51 (\textit{ΔmtbP}/\textit{ΔmttP1}/\textit{ΔmttP2}) and DJF52 (\textit{ΔmtmP}/\textit{ΔmtbP}), were prepared. These mutant
strain showed some growth defect with the DMA substrate. This observation indicates that there might be the cross-reactivity among the methyamine permeases. In order to fully understand the cross-reactivity among these permease, a quadruple knockout (ΔmtmP/ΔmtbP/ΔmttP1/ΔmttP2) needs to be prepared. In addition, a glycine betaine transporter, ProVWX, might have role in the transportation of trimethylamine as as it can bind to the active site in the same orientation as that of glycine betaine (Schiefner et al., 2004a). This possibility need to be addressed in the future. As an alternative approach, expression of these putative methyamine permeases in E. coli and an uptake assay with labelled substrate could answer our question directly.
Figure 20. Structure of A. monomethylamine, B. dimethylamine C. trimethylamine, and D. glycine betaine.
Figure 21. Structure of A. 4-aminobutyric acid (GABA), B. cis-3-aminocyclohexyl carboxylic acid, C. 3-aminobutyric acid, D. 5-aminopentanoic acid, E. 3-piperidinecarboxylic acid (nipecotic acid), and F. 3-aminopropanoic acid.
IV. REFERENCES


