Investigation of Large Protein Complexes Important for Methyl-Coenzyme M Reductase Activity and Function

By

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Abstract

Methyl-coenzyme M reductase (Mcr) is a key enzyme of methanogens that catalyzes the production or consumption of methane. Mcr contains a nickel tetrapyrrole cofactor (coenzyme F430) which has a crucial role in catalysis. The enzyme is only active when its cofactor is in the Ni(I) state, and it is extremely oxygen sensitive. Therefore, the activity is quickly lost without special precautions. Until now, the details on how Mcr gets activated intracellularly are not clear. Here we try to get a better understanding of the activation process. In vivo activation of Mcr involves two components, A2 and an iron-sulfur-containing large complex named A3a. In Dr. Duin's laboratory, native A3a from *Methanothermobacter marburgensis* was purified and its subunits were characterized. It was proposed that there is a core of activating proteins that include McrC, iron-sulfur flavoprotein (FFP), Mmp7 (methanogenesis marker protein 7), component A2, and ATP binding protein. Characterization of these proteins and pull-down experiments should provide a more complete picture of the actual activation complex and how it works. Genes from *Methanococcus maripaludis* were cloned in a pMEV4mTs plasmid with a strep Flag-Strep$_2$ tag and successfully expressed homologously in *M. maripaludis.* Recombinant proteins were purified with Strep-Tactin Superflow Plus resin. As expected, additional protein bands were detected on SDS-PAGE. The identity of these bands for recombinant Mmp7, A2 components, and McrC was verified by MALDI mass spectrometry. It is assumed that the additional bands are probably due to the direct interactions of recombinant protein with the other proteins involved in the Mcr activation. McrC with strep tag made a complex with Mmp7, Mmp3, Mmp17, and Mcr enzyme. The A2 component interacts with the Mcr. Mmp7 with strep tag made a complex with McrA, Mmp3, and McrB. Anaerobic purified recombinant Mmp7 has a dark brownish color. EPR and

UV–visible results showed that it contains an iron-sulfur cluster. The same was true for the A2 protein. FFP has a strong yellow color. The UV–visible and EPR results showed that a flavin and an iron-sulfur cluster are present in the sample.

Methanogenesis marker proteins are signature proteins of the archaea. In the *Methanosarcina acetivorans* C2 genome, there is an operon named *mmp* operon, which contains *A2* (*atwA)*, *mmp*3, *mmp6*, *mmp5*, *mmp15*, *mmp*17, and *mmp7*. It is assumed that these proteins form one complex and be involved in the activation and folding of Mcr. All genes were cloned in one pETDuet vector and expressed simultaneously in C41 (*E. coli*) cells. Since the A2 component can bind ATP, the A2 component was purified directly from cell extract with an ATP agarose column. Mass spectrometry results showed that except for Mmp3, all 7 proteins came down in one complex. Mmp3 is present, however, when the complex is isolated under the exclusion of air.

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List of Abbreviations

Mcr	Methyl coenzyme-M reductase
McrC	Methyl coenzyme M reductase, subunit C
McrD	Methyl coenzyme M reductase, subunit D
A2	Methyl coenzyme M reductase system, component A2
McrG	Methyl coenzyme M reductase, subunit gamma
McrA	Methyl coenzyme M reductase, subunit alpha
McrB	Methyl coenzyme M reductase, subunit beta
E. coli	*Escherichia coli*
M. acetivorans	*Methanosarcina acetivorans*
M. maripaludis	*Methanococcus maripaludis*
SAM	S-adenosylmethionine
Mmp	Methanogenic marker protein
HDR	Heterodisulfide reductase
M-CoM	M-CoM Methyl Coenzyme M
CW	Continuous wave
IPTG	Isopropyl-β-D-thiogalactoside
EPR	Electron paramagnetic resonance spectroscopy
FPLC	Fast protein liquid chromatography
UV-Vis	Ultraviolet-Visible spectroscopy
MS	Mass spectroscopy

SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
WT	Wild-Type
DIT	Dithionite
Fdh	Formate dehydrogenase
Fwd	Formyl methanofuran dehydrogenase
FFP	Iron-sulfur cluster protein
Mmp7	Methanogenesis marker 7
atwA	A2 gene
PCR	Polymerase chain reaction
Mvh	F420-non-reducing hydrogenase/methyl-viologen reducing hydrogenase
Mtr	Methyl-H4MPT/coenzyme M methyltransferase
Mer	Methylene-H4MPT reductase
Hmd	H2-forming methylene-H4MPT dehydrogenase
Mtd	F420-dependent methylene-H4MPT dehydrogenase
Mch	Methenyl-H4MPT cyclohydrolase
Ftr	Formylmethanofuran/H4MPT formyltransferase
Fwd	Formylmethanofuran dehydrogenase
HS-CoB	Coenzyme B
HS-CoM	Coenzyme M
MFR	Methanofuran
H4MPT	Tetrahydromethanopterin

Chapter 1 : Background and significance

1.1) Introduction

1.1.1) Methanogens

Methanogens produce methane through anaerobic respiration. Their initial substrate is CO_2, formate, CO, methanol, or acetate to produce ATP. All methanogenic archaea are strictly anaerobic and live in complete no-oxygen habitats, such as sediments, swamps, and the digestive tracts of animals.[1] Methanogens live in large quantities in low ferric, nitrate, and sulfate ions environments. In a rich ion environment, methanogens cannot compete with anaerobic bacteria. For example, sulfate-reducing bacteria grow in sulfate-rich marine environments. The initial substrate for sulfate-reducing bacteria is sulfate, a thermodynamically more favorable electron acceptor than CO_2.[1] Methanogens are considered a diverse group with over 50 species. The *Methanobacteriales* have rod-shaped cells and grow by CO_2 reduction, while a few species of the genus *Methanosphaera* have cocci-shaped cells and use methanol as a substrate. *Methanobrevibacter* species have short rod-shaped cells and use H_2 or formate to reduce CO_2.[1]

When methanogens use H_2 as a source of electrons to reduce CO_2, they are called hydrogenotrophic methanogenesis. They are assumed to be the oldest and most widespread form of methane production.[2] *Methanosarcinales* can use acetate as a substrate, called acetoclastic methanogenesis. They split acetate into a methyl group and an enzyme-bound carbonyl moiety. CH_3-H_4MPT is produced from the methyl group, which is later converted into methyl-CoM. In this case, the hydrogenotrophic pathway runs in reverse to produce CO_2 and electrons needed for the aceticlastic branch.[3] *Methanosarcinales*, *Methanobacteriales,* and *Methanomassiliicoccales* are methylotrophic methanogens. They can use C-1 compounds such as methanol or methyl-

amines as substrates.[4] In methylotrophic methanogens, one molecule of the C-1 compound is oxidized to be used as a source of electrons to reduce three C-1 compounds to methane.[3]

Methanogens have an essential role in the global carbon cycle. A large portion of the methane produced comes from methanogenesis.[5] Humans have an unquestionable role in increasing methane production by methanogens. Methanogens live in the ruminants of animals from livestock and produce a considerable amount of methane.[5,6] Methane is a greenhouse gas and has a warming effect on the atmosphere temperature with one of the highest percentages of radiative forcing.[7,8] Methane is a fuel and can be used to replace fossil fuels.

1.1.2) Global Carbon cycle

Carbon constantly cycles back and forth between the atmosphere to the land and ocean and then back into the atmosphere. Earth is a closed system, and the amount of carbon in this closed system is constant. In lands, carbon accumulates in soils, and terrestrial biomass in sediment in inland waters such as lakes, streams, and rivers. Inland waters transport, mineralize and bury carbon. After soil erosion, carbon sinks shift from soils and terrestrial biomass to inland waters. Terrestrial photosynthesis takes up carbon from the atmosphere and stores it in terrestrial biomass. Respiration release carbon from terrestrial biomass into the atmosphere.[9] Light natural gases such as methane, carbon dioxide, and water make solid crystals under the ocean called Gas hydrates. Methane hydrates release about 1Gt of methane per year.[10] Humans also play a significant role in the carbon cycle (Figure 1). Livestock and rice cultivation, deforestation and shifting cultivation, agricultural waste, mining and use of fossil fuels, and microbiological decomposition of domestic waste and sewage are important sources of anthropogenic emissions. For example, only about 30% of methane in the atmosphere is natural.[11]

In the global carbon cycle, a large section of the methane produced comes from methanogenesis (Figure 1). Methanogenesis makes about 1 Giga ton (GT) of methane yearly. In the rumen of ruminants, there is a syntrophic association between anaerobic fungi, anaerobic bacteria, protozoa, and methanogenic archaea. Anaerobic fungi, anaerobic bacteria, and protozoa hydrolyze large molecules such as biopolymers to monomers and lipids to glycerol and fatty acids and ferment them to CO_2 and acetic acids. The methanogens use CO_2, H_2, and acetate and produce methane.[5,6]

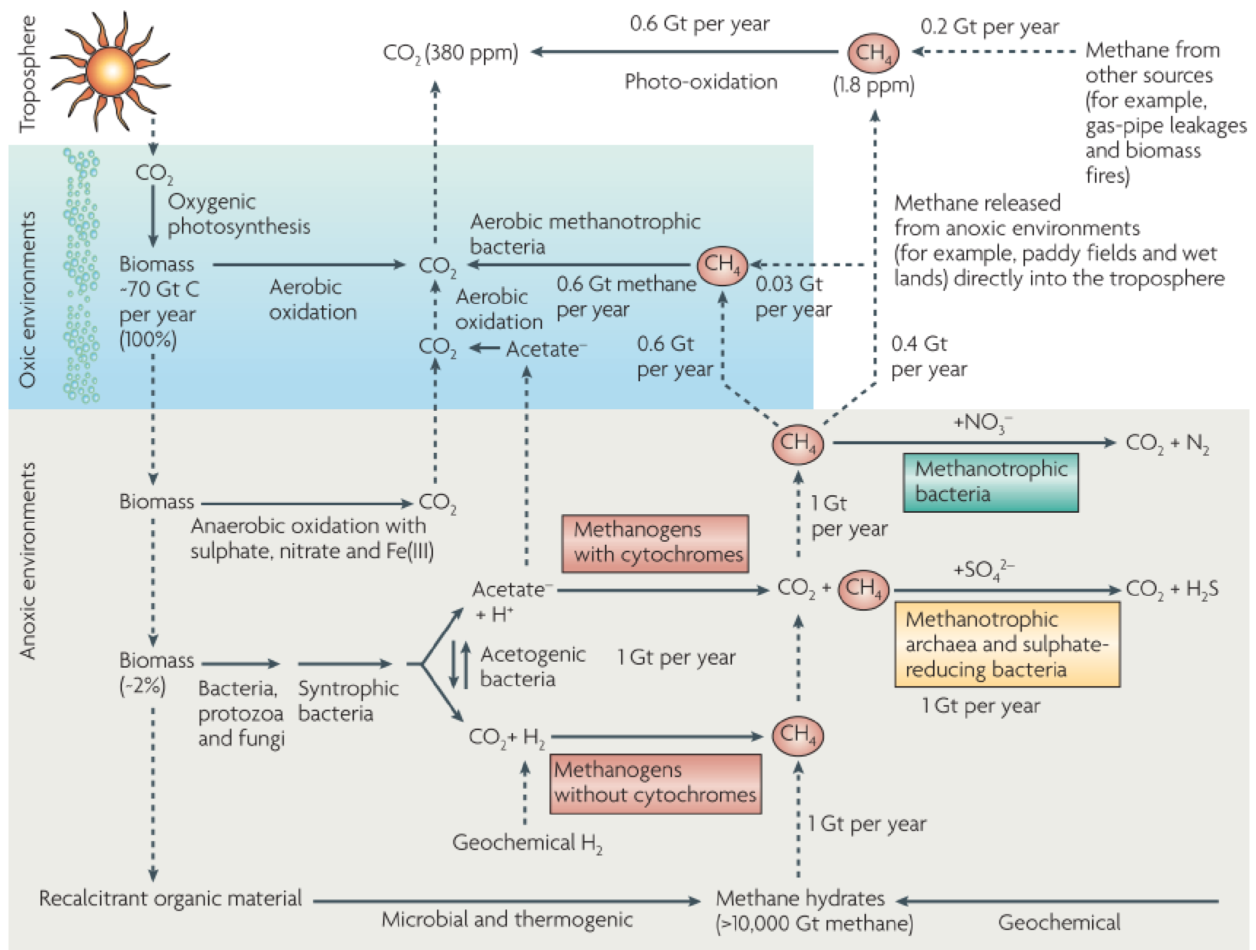

Figure 1) In The global carbon cycle, methanogenic archaea produce about 1 Giga ton (Gt) of methane per year from CO_2 and acetate in anaerobic environments such as swamps and the intestinal tracts of ruminants and termites. Methane hydrates release about 1Gt of methane yearly.[5] The microbiological decomposition of sewage and waste produces methane.[11] Aerobic bacteria oxidized 0.6 Gt methane to CO_2 per year. Reproduced from (Thauer et al. 2008) with permission from Springer Nature Copyright © 2008.

1.1.3) Greenhouse gases (GHGs)

The sun is the source of energy for the earth. The earth absorbs some part of the energy, and the rest of the energy reflects into space. The Earth's average surface temperature and climate depend on this balance. Radiative forcing is an essential indicator of how gas in the atmosphere influences the balance of incoming and outgoing energy. The radiative forcing of some gases is shown in Table 1 and Figure 2. Some of the gases have a warming effect and raise the temperature. They are called positive forcing, some have a cooling effect and are called negative forcing.[8,12]

CO_2 and methane are two greenhouse gases with the highest percentage of radiation forcing. They are produced by natural and anthropogenic processes. Concentrations of CO_2 and methane have increased continuously in recent centuries due to rapid industrialization. For example, the estimated global concentration of CO_2 in the atmosphere was around 280 ppmv in 2009 (parts per million measured as volumes) and increased to 392 ppmv in 2011.[7]

Table 1) Percentage of relative radiative forcing of some gases. The table is republished from (Vallero and Letcher 2013) with permission from Elsevier Copyright © 2013

Gas	Percentage of relative radiative forcing
Carbon dioxide, CO_2	64
Methane, CH_4	19
Halocarbons (predominantly Chlorofluorocarbons, CFCs)	11
Nitrous oxide, N_2O	6

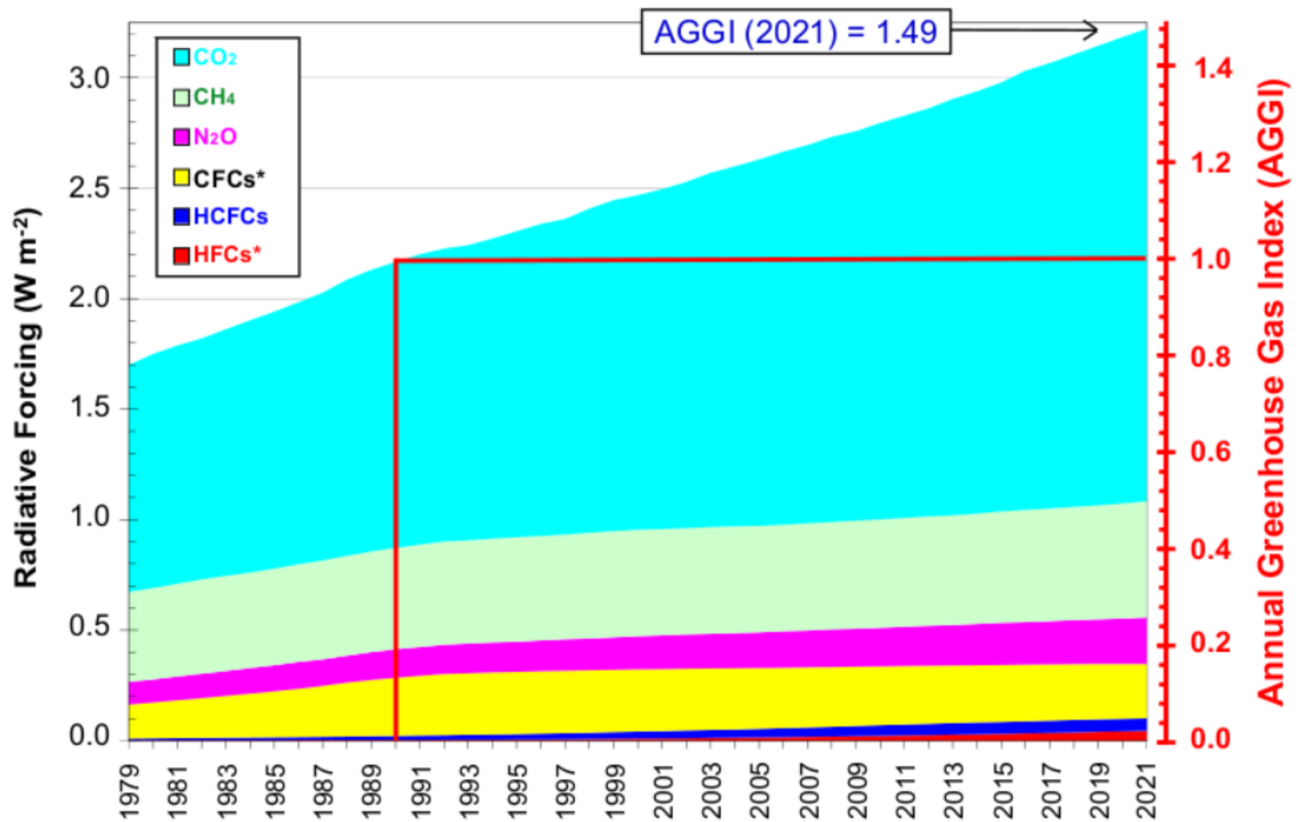

Figure 2) The energy imbalance in the atmosphere is shown with radiative forcing (watts per square meter), and the size of influence on climate changes is shown with the greenhouse gas index. In 2019, there was a 45 percent increase in radiative forcing compared to 1990. CO_2 has the most significant percentage of the radiative forcing from 1990. (Taken from: https://web.archive.org/web/20210513163545/https://gml.noaa.gov/aggi/)

1.1.4) Hydrogenotrophic pathway or the 'Wolfe cycle.'

Methane formation in hydrogenotrophic methanogenesis starts with a two-electron reduction of CO_2 and the production of formyl-MFR by formyl-MFR dehydrogenase (***a***; Fwd). Methanofuran (MFR) is the first C1 acceptor molecule. Coenzymes such as methanofuran (MFR), tetrahydromethanopterin (H_4MPT), and CoM-SH work as C-1-unit carriers or vectors. H_2 and formate are electron sources. These electrons cause a sequential reduction of CO2 and methane production (Figure 3). CO2 binds to the amino nitrogen atom of MFR and H_2 reduces it to formyl. The Formyl group is subsequently transferred to the tetramethanopterin (H_4MPT) by formylmethanofuran-H_4MPT formyl transferase (***b***; Ftr) and produces formyl-H_4MPT. H_4MPT is the second C1 acceptor molecule and carbon carrier. The Formyl group is converted to a methenyl group by 5,10-methenyl tetrahydromethanopterin cyclohydrolase (***c***; Mch) and produces (methylene-H_4MPT (HC≡H_4MPT)). HC≡H_4MPT is reduced to HC=H_4MPT by F_{420}-dependent methylenetetrahydromethanopterin dehydrogenase (***d***; Mtd). Methylene-H_4MPT (HC=H_4MPT) is reduced to methyl-H_4MPT (CH_3-H_4MPT) by Methylenetetratetrahydromethanopterin reductase (***e***; Mer) with coenzyme $F_{420}H_2$. The methyl group is transferred from methyl-H_4MPT to CoM-SH by coenzyme M methyl transferase (***f***; Mtr) and this reaction is an exergonic reaction ($\Delta G°' = -30$ kJ/mol). The reaction is coupled to H^+ or Na^+ transport over the cell membrane by the membrane bound Mtr complex. Methyl-coenzyme M is reduced by CoB-SH to methane and the heterodisulfide CoM-S-S-CoB, a reaction catalyzed by methyl-coenzyme M reductase (***g***). It is an exergonic reaction ($\Delta G°' = -30$kJ/mol). Heterodisulfide CoM-S-S-CoB is reduced with H_2 by the hydrogenase/heterodisulfide reductase complex (***h***; Hdr). It is an exergonic reaction ($\Delta G°' = -55$kJ/mol). This reaction can be coupled with the endergonic ferredoxin reduction when the hydrogenase/Hdr complex is present as a cytosolic complex. It can generate an H^+ or Na^+ gradient

when it is present as a membrane-bound complex. In some methanogens, the hydrogenase/Hdr forms a complex with Fwd and Fhd (Formate dehydrogenase, ***k***), and the first and the last step of the pathway are directly coupled which led to Rolf Thauer proposing the name 'Wolfe Cycle' in honor of Ralph Wolfe, who passed away on March 26, 2019, at the age of 97.[13,14]

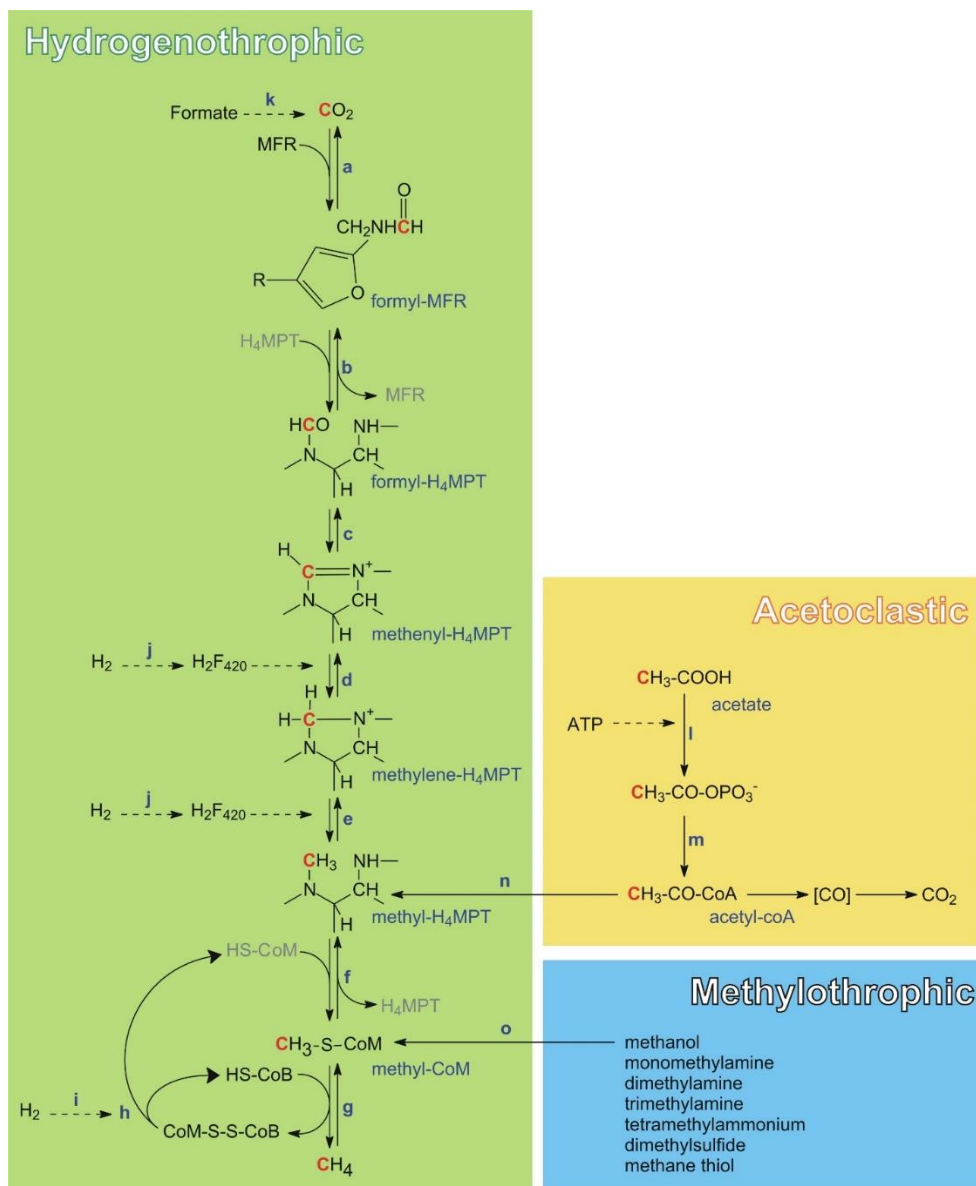

Figure 3) Methane formation pathways. H_2 and formate are electron sources. These electrons cause a sequential reduction of CO_2 and the production of methane. H_4MPT, tetrahydromethanopterin; MFR, methanofuran; HS-CoM, coenzyme M; HS-CoB, coenzyme B. Enzymes: **a**, formylmethanofuran dehydrogenase (Fwd or Fmd)); **b**, formylmethanofuran/H_4MPT formyltransferase (Ftr); **c**, methenyl-H_4MPT cyclohydrolase (Mch); **d**, F_{420}-dependent methylene-H_4MPT dehydrogenase (Mtd) or H_2-forming methylene-H_4MPT dehydrogenase (Hmd); **e**, methylene-H_4MPT reductase (Mer); **f**, methyl-H_4MPT/coenzyme M methyltransferase (Mtr); **g**, methyl-coenzyme M reductase (Mcr or Mrt); **h**, heterodisulfide reductase (Hdr); **i**, F_{420}-non-reducing hydrogenase/methyl-viologen reducing hydrogenase (Mvh); **j**, F_{420}-reducing hydrogenase; **k**, formate dehydrogenase (Fdh); **l**, Acetate kinase (Ack): **m**, phosphotransacetylase (Pta) (**l+m**, thiokinase in Methanosaeta spp.); **n**, carbon-monoxide dehydrogenase (Cdh)/acetyl-CoA synthase (Acs); **o**, methyl transferases.[13,14]

1.1.5) About Methyl-coenzyme M reductase (Mcr)

Methyl-coenzyme M reductase (Mcr) catalyzes the last step of anaerobic methane production in methanogenic archaea and is involved in biological anaerobic methane production. In methanogenic archaea, Mcr converts the two substrates, methyl-coenzyme M (CH_3-S-CoM) and coenzyme B (HS-CoM), into methane and a heterodisulfide (CoB-S-S-CoM).[15] (Figure 4)

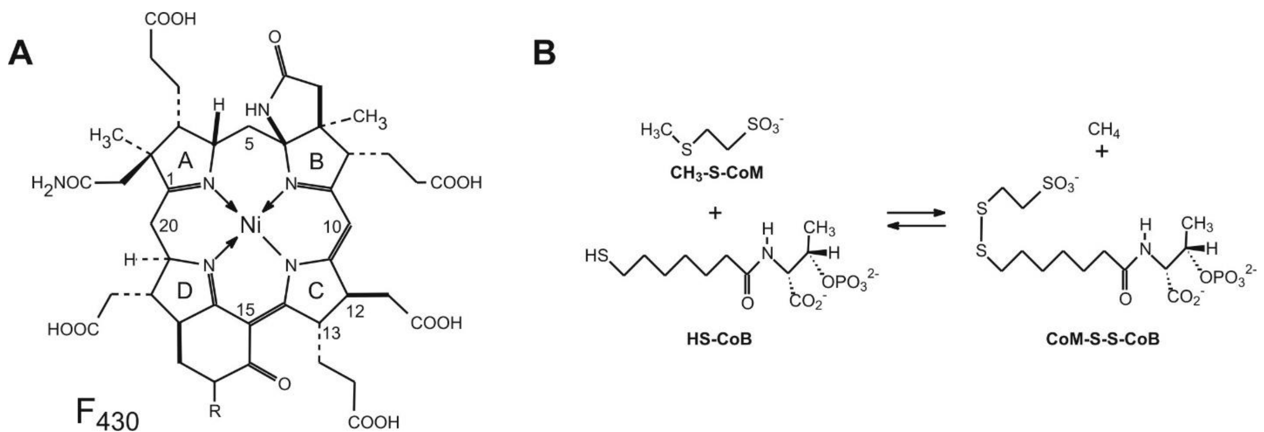

Figure 4) (**A**) The cofactor, factor 430 (F_{430}), found in the active site of Mcr (**B**) Reaction catalyzed by Mcr. Mcr converts the two substrates, methyl-coenzyme M (CH_3-S-CoM) and coenzyme B (HS-CoM), into methane and a heterodisulfide (CoB-S-S-CoM).

Upon opening the cells, the Mcr enzyme losses its activity even in a strictly anaerobic environment due to the very negative electron redox potential of Ni(I)F_{430}. This is mainly because the hydrogenases under low H_2 concentrations will start to oxidize the cytosol resulting in inactivation. When Mcr loses its activity there is no way to turn it to the active form again *in vitro*.

This problem limits the study of Mcr. Every time researchers try to study the enzyme, freshly purified Mcr is needed. The native organism needs to be grown and then anaerobically harvested and purified. It is a very time-consuming and sensitive process. Even a little bit of oxygen leaking during harvest or purification can cause a loss of activity.

1.1.6) Mcr structure

Methyl-coenzyme M reductase (Mcr) was purified for the first time from *Methanobacterium thermoautotrophicum*, strain ΔH (later renamed *Methanothermobacter thermoautotrophicus*) by Wolfe and coworkers in 1981. The native protein has a molecular weight of 300 kiloDaltons and has a hexameric structure (α_2 β_2 γ_2).[16] Eighteen years later, the crystal structure revealed that two nickel-containing tetrahydrocorphinoids (coenzyme $F_{430)}$ are present and are coordinated by either α, α′, β, and γ, or the α, α′, β′, and γ′ subunits. Mcr has two separate active sites, and a long narrow channel makes each active site accessible for both substrates. F_{430} is present at the end of the channel).[17,18]

Several states for Mcr have been revealed, but three major forms can be found in the intact cells or purified enzymes (Figure 5). The active form of the enzyme is Mcr_{red1} with the nickel of F_{430} in the +1 oxidation state. It is paramagnetic and shows a signal in electron paramagnetic resonance (EPR) spectroscopy. The signal is close to the axial with $g_1 = 2.25$; $g_2 = 2.07$; $g_3 = 2.06$ (Figure 6). It has a greenish color and a maximum absorption at 385 nm and a broad band at 720 nm in the ultraviolet-visible (UV–vis) spectrum (Figure 7). A second form is $Mcr_{ox1,}$ with the nickel in the +3 oxidation state. It is also paramagnetic and shows an axial EPR signal with $g_1 = 2.2310$; $g_2 = 2.1667$; $g_3 = 2.1532$ (Figure 6). It has an orange color with maximum absorption at

415 nm and a small band at 650 nm (Figure 7). The third form is $Mcr_{silent,}$ with the nickel in the +2 oxidation state. This form does not show an EPR signal (EPR inactive or EPR silent). It has a bright yellow color with absorption at a peak at 420 nm and a shoulder at 445 nm (Figure 7).[19]

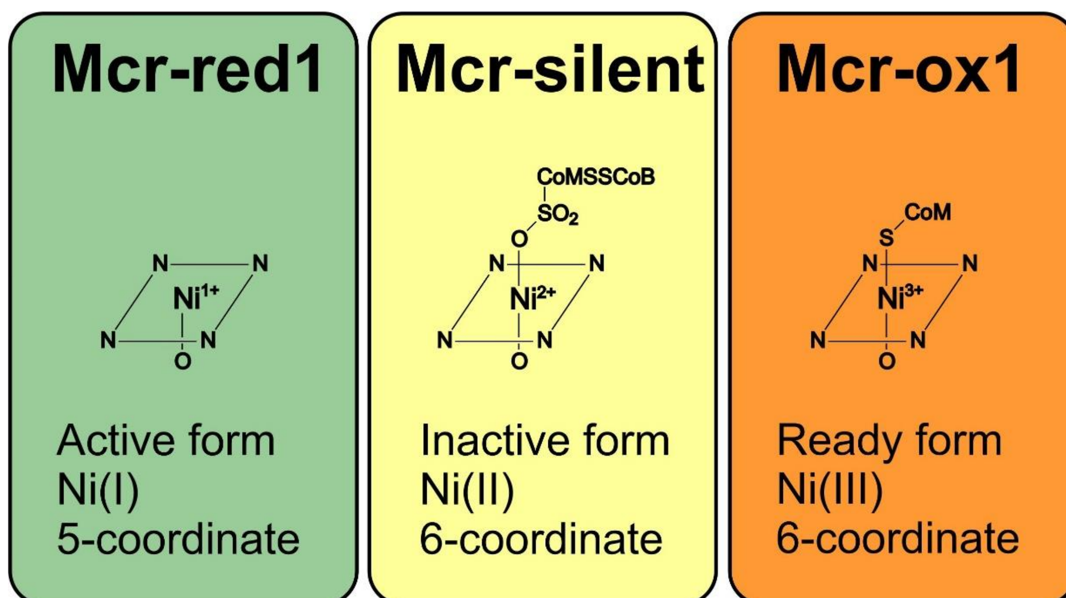

Figure 5) Major forms of Mcr: The active form of the enzyme is Mcr_{red1} with the nickel of F_{430} in the +1 oxidation state. A second form is $Mcr_{ox1,}$ with the nickel in the +3 oxidation state. The third form is $Mcr_{silent,}$ with the nickel in the +2 oxidation state.

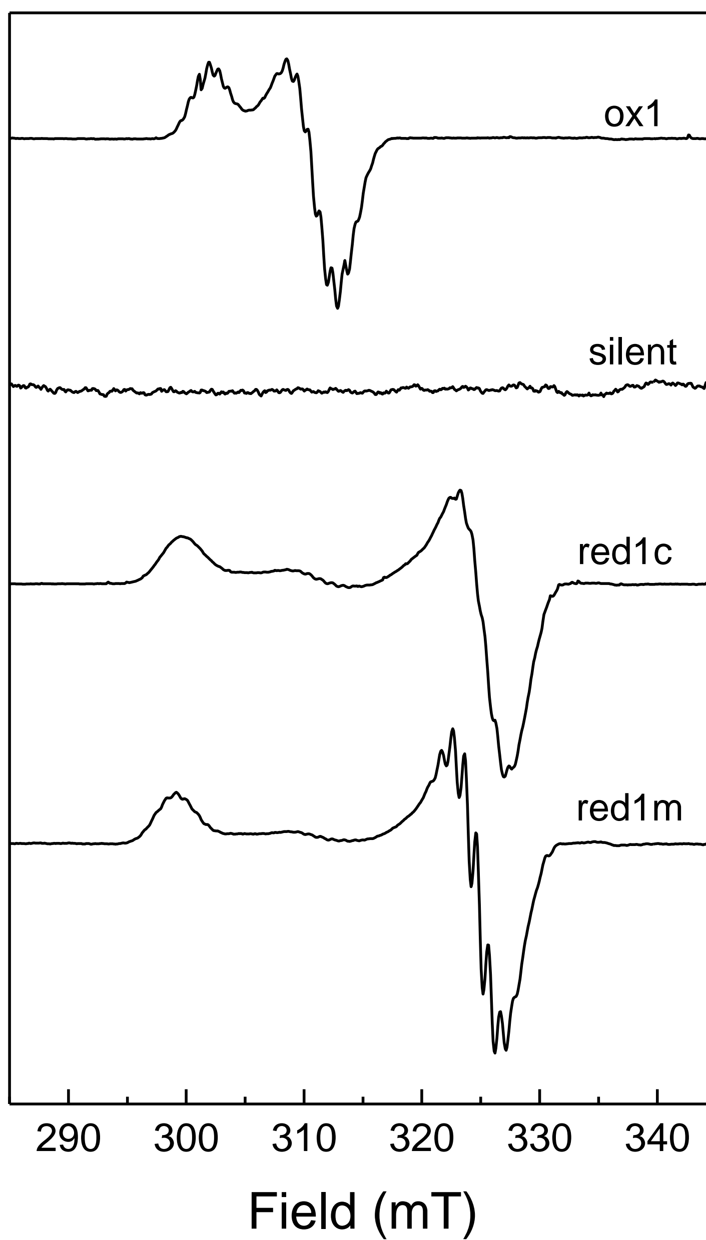

Figure 6) EPR spectra of Mcr. Mcr_{red1} is paramagnetic and shows a signal in electron paramagnetic resonance (EPR) spectroscopy. The signal is close to the axial with g1 = 2.25; g_2 = 2.07; g_3 = 2.06. Mcr_{ox1} shows an axial EPR signal with g_1 = 2.2310; g_2 = 2.1667; g_3 = 2.1532. Mcr_{silent} does not show an EPR signal.

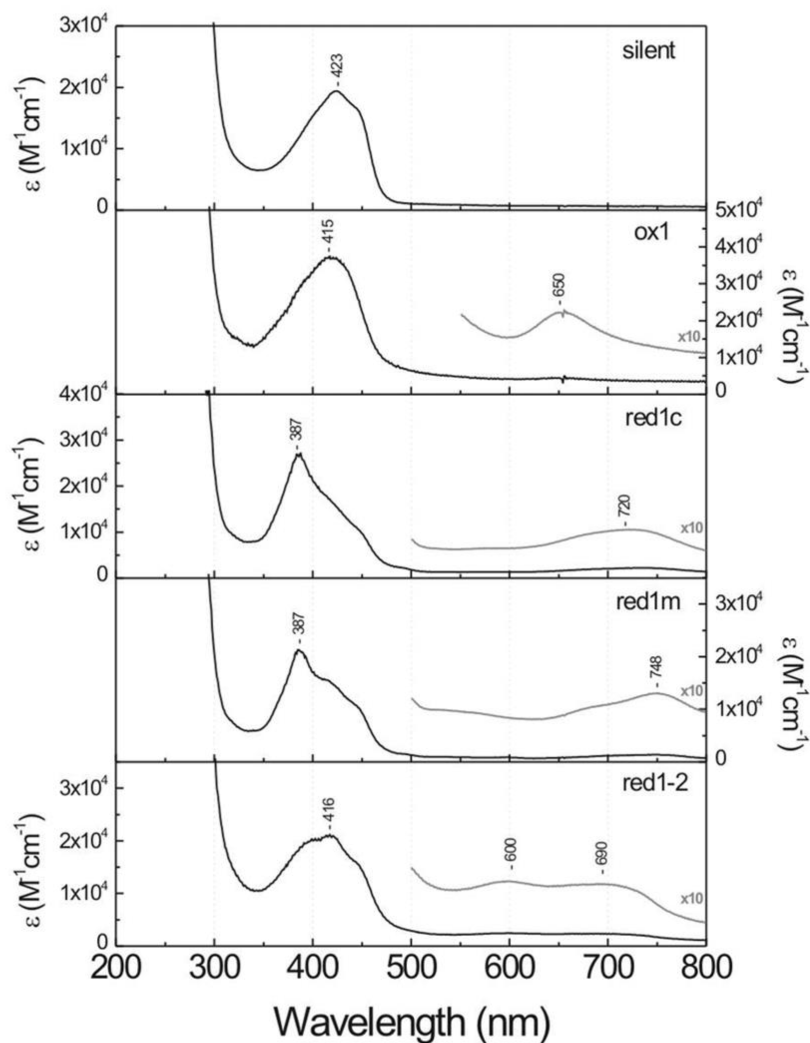

Figure 7) Mcr_{red1} has a greenish color and a maximum absorption at 385 nm and broadband at 720 nm in the Ultraviolet-visible (UV–Vis) spectrum. Mcr_{ox1} has an orange color with maximum absorption at 415 nm and a small band at 650 nm. Mcr_{silent} has a bright yellow color with absorption at a peak at 420 nm and a shoulder at 445 nm.

Mcr_{red1} is not stable but can be stabilized with methyl-S-CoM or HS-CoM, and these forms are named Mcr_{red1m} and Mcr_{red1c}, respectively. In both, Ni (+1) has five ligands. Ni has four equatorial ligands with four nitrogens of F_{430} and one distal axial ligand with oxygen from the side chain of a glutamine residue. When Mcr_{red1c} and Mcr_{ox1} are exposed to the air, $Mcr_{red\text{-}1silent}$ and $Mcr_{ox\text{-}1\ silent}$ are made. Both are EPR silent and have Ni in the +2-oxidation state. Ni in $Mcr_{ox1\text{-}silent}$ is a six-coordinated complex, and the sulfur of the thiol group from coenzyme M is the sixth ligand in the proximal position of the complex. Mcr_{ox1} has the same coordination around Ni, but the oxidation state of Ni is +3. Ni in Mcr_{silent} is six coordinated complexes, and oxygen from the sulfonate group of CoM-S-S-CoB is the other proximal ligand (Figure 5).[20] The Mcr_{ox1} form (Ni(III)) can be directly converted into the red1 form (Ni(I)) by incubation with Ti(III) citrate at pH 9.0.[21] The E_m for Ti(III) citrate decreases linearly from -0.48 V at pH 7 to nearly - 0. 70 V at pH 10.[22]

1.1.7) Reaction mechanism of Mcr

Mcr catalyzes the final step of methane production. X-ray structures of Mcr-Ni(III)-methyl and Mcr-Ni (II) revealed that both substrates methyl-S-CoM and CoB-SH enter the active site through the same channel (Figure 8). There is a hydrophobic cavity above the F430. Methyl-S-CoM probably enters the active site first because its binding site is more buried and then the binding site of CoB-SH. The role of nickel is very critical in catalytic mechanisms for Mcr.[18]

For methane formation, a carbon isotope effect ($^{12}CH_3$-S-CoM/$^{13}CH_3$-S-CoM) was observed, and it showed that the rate-limiting step is the cleavage of the C−S bond in the substrate Me-S-CoM.[23] Three proposed mechanisms exist (Figure 9).[24] The first intermediates are different in the mechanisms. In the first proposed mechanism, Ni (I) does a nucleophilic attack on the methyl group of methyl-S-CoM and produces a methyl-Ni(III) intermediate. In the second proposed mechanism, Ni(I) attacks the sulfur atom of methyl-S-CoM and produces a methyl radical and Ni(II)-thiolate complex. Cleavage of the methyl-sulfur bond is homolytic.[18] In the third proposed mechanism, Ni (I) does a nucleophilic attack on the sulfur atom of methyl-S-CoM and produces an anionic methyl group and Ni(III)-thiolate complex. In 2016, Wongnate et al. were able to trap reaction intermediates and show the nickel was in the Ni(II) state. This information supports reaction mechanism II.[24]

For a complete description: In the first step, Ni(I) attack on the sulfur of methyl-S-CoM leads to homolytic cleavage of the C-S bond and generation of a methyl radical and a Ni(II)-thiolate (Mcrox1-silent). Next, H-atom abstraction from CoB-SH generates methane and the CoB-$S^{\bullet}$ radical, which in the third step combines with the Ni-bound thiolate of CoM to generate the Ni(II)-disulfide anion radical. Then, one-electron transfer to Ni(II) generates Mcr_{red1} and the heterodisulfide (CoB-S-S-CoM) product, which dissociates, leading to ordered binding of methyl-

S-CoM and CoB-SH and initiation of the next catalytic cycle. All radical species are expected to be unstable and have not been detected.[18]

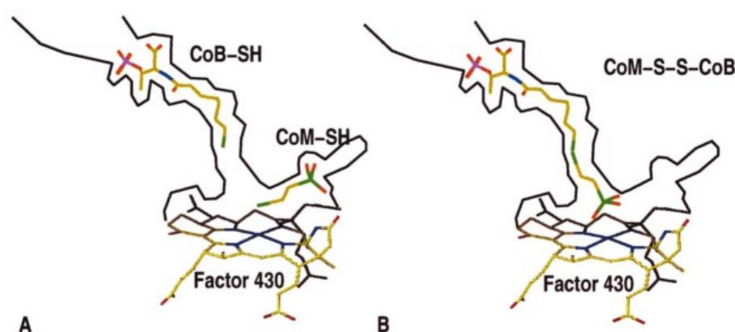

Figure 8) A: The crystal structures of the Mcr-ox1-silent state of Mcr showed that the active site was occupied with CoB-SH and HS-CoM. B: The crystal structures of the Mcr-silent state showed the active site was occupied with CoM-S-S-CoB.[15] The figure was reproduced from Grabarse 2001 with permission from Elsevier Copyright © 2001 Academic Press.

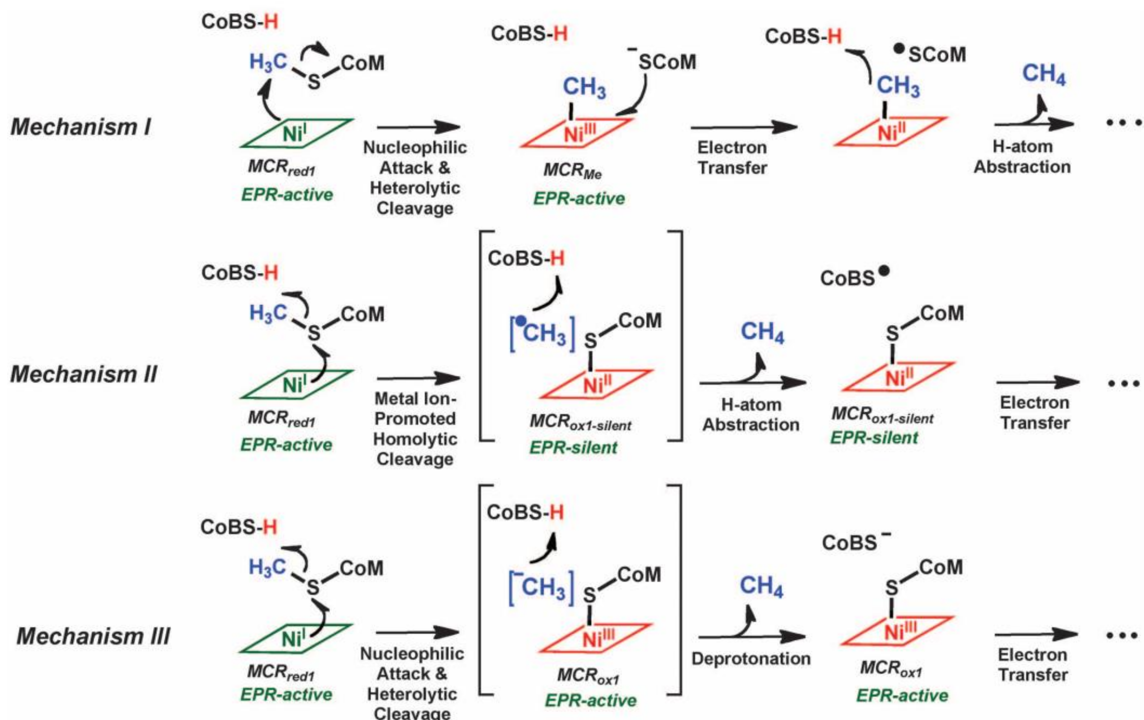

Figure 9) Three mechanisms were proposed. In the first proposed mechanism, Ni (I) does a nucleophilic attack on the methyl group of methyl-SCoM, producing a methyl-Ni(III) intermediate. In the proposed mechanism II, Ni(I) attacks the sulfur atom of methyl-S-CoM and produces a methyl radical and Ni(II)-thiolate complex. Cleavage of the methyl-sulfur bond is homolytic. In the third proposed mechanism, Ni (I) does a nucleophilic attack on the sulfur atom of methyl-S-CoM and produces an anionic methyl group and Ni(III)-thiolate complex.[24] Reprinted with permission from AAAS from Wongnate et al. 2016.

1.1.8) Post-translational modification

There are four to six post-translationally modified amino acids within the active-site pocket of Mcr of methanogens. *N*-methylhistidine, *S*-methylcysteine, 2-(*S*)-methylglutamine, 5-(*S*)-methylarginine, didehydroaspartate, thioglycine, and 6-hydroxy-tryptophan have been detected in methanogenic Mcrs. S-adenosylmethionine (SAM) probably supplies the methyl groups of the methylated residues.[25] The methylations of arginine at C5 and glutamine at C2 are very rare and only found in Mcr. These methylations are challenging and will require specialized radical SAM enzymes.[26]

5-(S)-methylarginine: In 2022, Layer and coworkers discovered that the enzyme glutamine C-methyltransferase is responsible for the post-translational modification of the 2-(*S*)-methylglutamine residue in Mcr. It is a cobalamin-dependent radical SAM enzyme. The methylation is done by radical mechanism rather than S-adenosyl-L-methionine-dependent methylation with S_N2 mechanism. A radical SAM enzyme contains a [4Fe-4S] cluster. Three cysteine residues of the enzyme are coordinated to the cluster. A SAM molecule is also coordinated to the cluster during the reaction. The cluster can be in the $[4Fe\text{-}4S]^{1+}$ or $[4Fe\text{-}4S]^{2+}$ forms. An electron from the reduced form of $[4Fe\text{-}4S]^{1+}$ transfers to the SAM molecule. Methionine and a 5′-deoxyadenosine radical (DOA) are produced from the cleavage of the SAM molecule. The radical DOA removes hydrogen from a substrate and makes a radical substrate. The radical substrate reacts with methylcob(III)alamin(MeCbl) and produces a methylated product and cob(II)alamin. An electron reduces cob(II)alamin to cob(I)alamin. A SAM molecule reacts with cob(I)alamin and regenerates MeCbl.[26] (Figure 10)

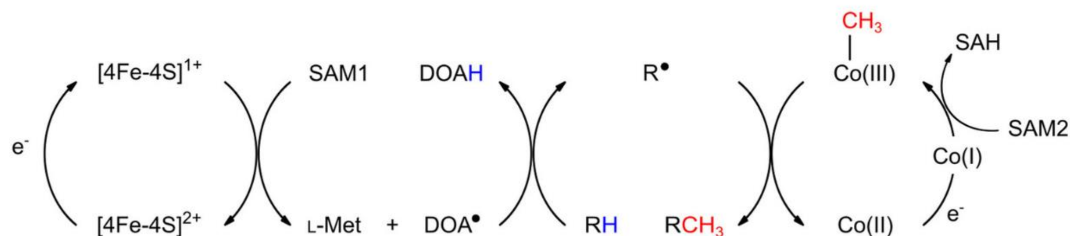

Figure 10) Glutamine C-methyltransferase enzyme is responsible for the methylation of glutamine and produces 2-(*S*)-methylglutamine. The enzyme contains a cluster, and the same molecule is coordinated to the cluster. An electron reduces the $[4Fe\text{-}4S]^{2+}$ cluster to $[4Fe\text{-}4S]^{1+}$. Then electron transfers from the cluster to the SAM molecule and produces l-methionine and a 5′-deoxyadenosyl radical (DOA) from the cleavage of the SAM molecule. The radical DOA removes hydrogen from a substrate and makes a radical substrate. The radical substrate reacts with methylcob(III)alamin(MeCbl) and produces methylated product and cob(II)alamin. An electron reduces cob(II)alamin to cob(I)alamin. A SAM molecule reacts with cob(I)alamin and regenerates MeCbl.[26] The figure was republished from Gagsteiger et al. 2022 with permission from John Wiley and Sons.

Thioglycine: For this conversion, two genes *ycaO* and *tfuA* (locus MA0165/MA0164 in *M. acetivorans*), are required.[27] Thioamides are not abundant in living organisms, and most of them have a bacterial origin. Some of them result from post-translational modification after synthesis by the ribosome, and some of them are unusual non-ribosomal peptides. The YcaO-dependent synthesis usually needs a protein partner. TfuA and YcaO proteins are encoded to the thioviridamide pathway.[28] The proposed mechanism for forming the thioamide bond with YcaO is shown in Figure 11.[29] It was suggested that this modification could help keep residues near the active site in the proper structural conformation to avoid Mcr destabilization.[28]

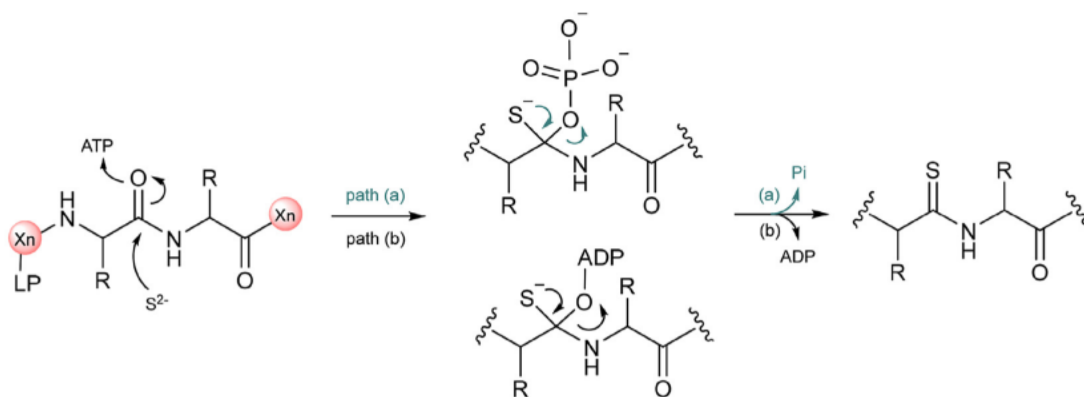

Figure 11) Proposed mechanisms for the formation of thioamide bonds with YcaO.[29] The figure was reprinted from (Z. Zhong et al. 2020), which is an open access article under the CC BY-NC-ND license

5-(S)-methylarginine: 5-(*S*)-methylarginine post-translational modification is almost available in all Mcrs from methanogenic archaea. But it is not available in Mcr from the methanotrophic archaea of the ANME-1.[30] Mmp10 with locus MA4551 is responsible for the modification installment of 5-(*S*)-methylarginine. It is a radical *S*-adenosylmethionine methyltransferase.[27] *mmpX* is the gene for the Mmp10 protein and is located next to *the mcr* operon in methanogenesis.[31] The rate of methane formation by cells decreased by 40 to 60% when *mmpX* was knocked out on some growth substrates but not all of them. It was proposed that the lack of methylation might result in misfolding, lower catalytic activity, and loss of stability of Mcr.[31]

S-methylcysteine: McmA with locus MA4545 responsible for *S*-methylcysteine in *M. acetivorans* is a SAM-dependent methyltransferase. The C-terminal domain of McmA has a conserved sequence for SAM binding.[27] *N*-methylhistidine post-translational modification is available in all analyzed Mcrs, which is crucial for all Mcr. *N*-methylhistidine helps coenzyme B bind to its binding site more effectively. Methylated nitrogen could make stronger hydrogen bonds with the phosphate group of coenzyme B.[30]

Didehydroaspartate: A post-translationally modified amino acid of didehydroaspartate near the thioglycine, as revealed by mass spectrometry and high-resolution X-ray crystallography, was reported by Shima and coworkers in 2016. Mcr I and II from *M. marburgensis* and Mcr from *Methanosarcina barkeri* contain the Didehydroaspartate post-translational modification. Some hydrogenotrophic methanogenic archaea have two Mcr isoenzymes, Mcr I and Mcr II. 60–70 % of their primary structures are identical.[25]

6-Hydroxy-tryptophan: This posttranslational modification was detected in Mcr III from *methanotorris formicicus* by Shima and coworkers in 2017. Three types of Mcr are found in *m. formicicus*. They have similar structures and active site architectures. But they differed slightly in protein surface regarding loop geometries and electrostatic properties. Mcr I, II, and III almost have the same post-translational modifications, but Mcr type III lacks methyl-cysteine but contains 6-hydroxy-tryptophan.[32]

Post-translational modifications are an energetically expensive process for archaea. When active sites cannot be optimized more with amino acid replacement, post-translational modification might be favorable for the anaerobic methane oxidation reaction. The rate-limiting step in methane formation from H_2 and CO_2 is the Mcr reaction. Any improvement in the catalytic power of Mcr increases the rate of methane formation.[25]

1.1.9) Heterodisulfide reductase (Hdr)

Hdr is a necessary enzyme in the Wolfe Cycle and is responsible for the reduction of heterodisulfide (CoM-S−S-CoB) to coenzyme M (CoM-SH) and coenzyme B (CoB-SH). This reaction enables the continuous regeneration of the intermediates (coenzyme B, coenzyme M) for the Wolfe cycle. The CoM-SH product is converted into CH_3-S-CoM by the methyltransferase (Mtr). X-ray crystal structures of the complex of heterodisulfide reductase (HdrABC) with [NiFe]-hydrogenase (MvhAGD) from the thermophilic methanogenic revealed a dimer of HdrABC-MvhAGD (Figure 12). HdrB contains two non-identical noncubane [4Fe-4S] clusters, and they are associated with the disulfide bond dissociation of CoM-S−SCoB.[33] (Figure 13)

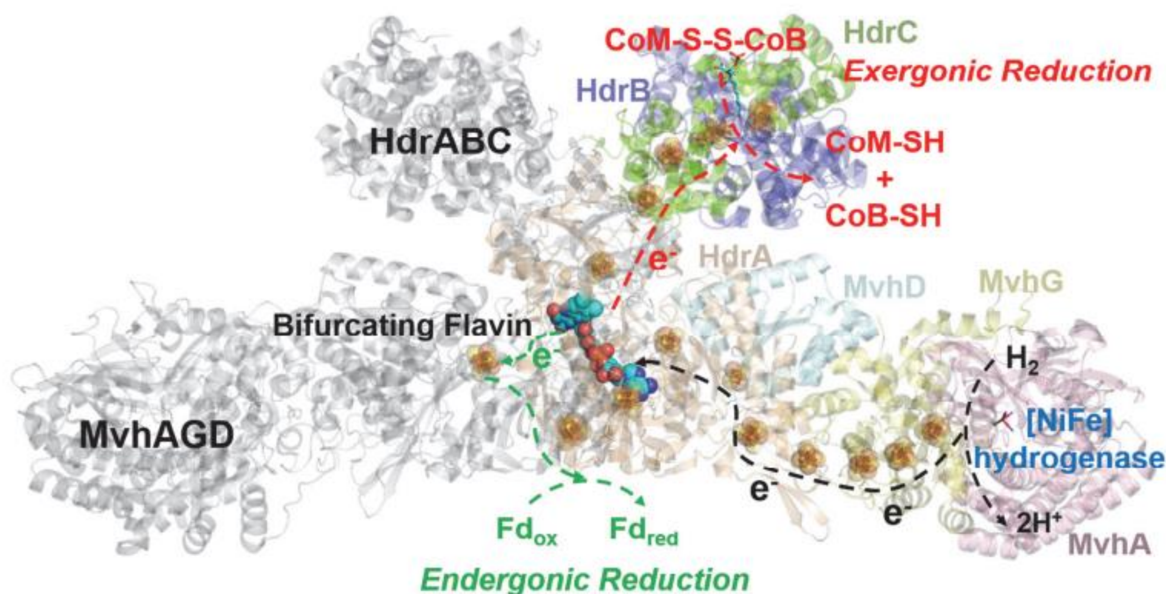

Figure 12) X-ray crystal structures of the complex of heterodisulfide reductase (HdrABC) with [NiFe]-hydrogenase (MvhAGD) from the thermophilic methanogenic revealed a dimer of HdrABC-MvhAGD .[33] Heterodisulfide CoM-S-S-CoB reduces with H_2 by hydrogenase–heterodisulfide reductase (Hdr) complex. It is an exergonic reaction ($\Delta G^{\circ\prime}=-55$kJ/mol). This reaction is coupled with the endergonic reaction of ferredoxin reduction with H_2 ($\Delta G^{\circ\prime}= +16$ kJ/mol). The reduced ferredoxin re-starts the Wolfe cycle.[13,14] Reprinted with permission from Wu and Chen 2022. Copyright 2022 American Chemical Society.

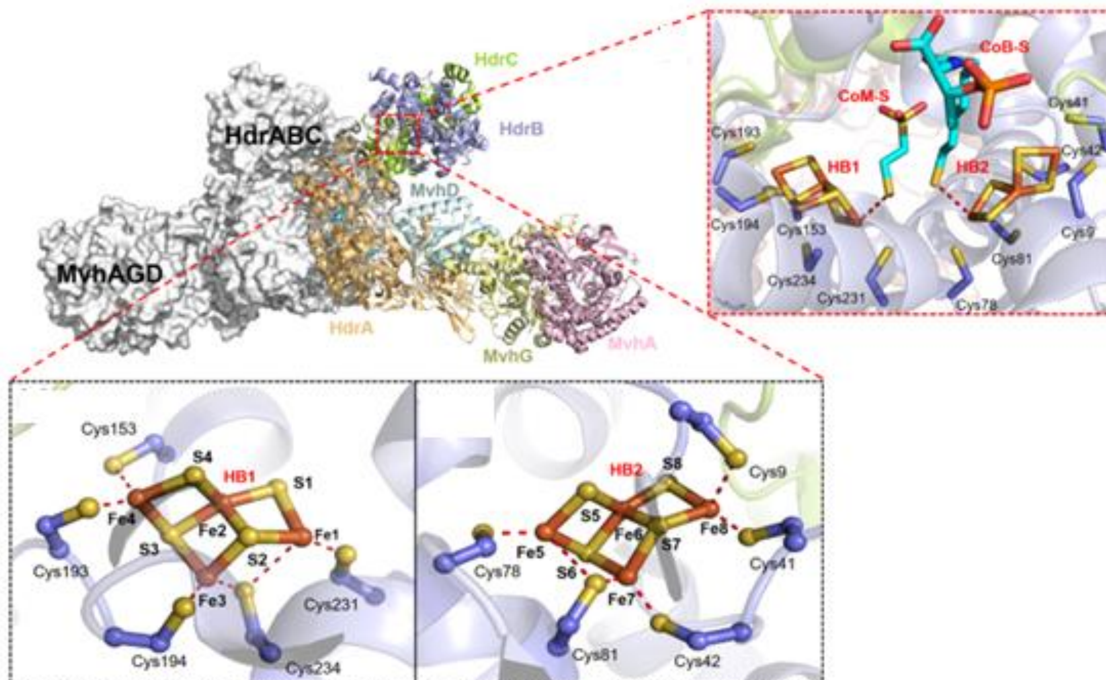

Figure 13) Two non-identical uncommon noncubane [4Fe-4S] clusters ((HB1 and HB2) were detected in HdrB. They are associated with the disulfide bond dissociation of CoM-S−S-CoB. Reprinted with permission from Wu and Chen 2022. Copyright 2022 American Chemical Society.[33]

Hdr can be found in different complex forms. The classic 4-column-step purification procedure for the *M. marburgensis* enzyme provides the Hydrogenase/Hdr complex.[34,35] This can be further split into two separate enzymes if needed When the protein is tagged with a His-tag and only one column step is used the Hydrogenase/Hdr complex is part of a bigger complex that also includes formate dehydrogenase (Fdh) and formyl methanofuran dehydrogenase (Fwd).[36] This was shown for the enzyme purified from *M. maripaludis*. Its composition will change whether the cells are grown on hydrogen or formate. When grown on formate the Hydrogenase is not part of the complex.[37]

Duin and coworkers discovered that the Hydrogenase/Hdr/Fdh/Fwd complex might be part of a bigger complex that is responsible for the activation of Mcr and possibly also for the folding of Mcr.[38] The complex will be described further below. Some of the subunits are so-called methanogenesis marker proteins, which will be discussed first.

1.1.10) About Methanogenesis marker cluster

Some specific proteins are unique to methanogens. They are named signature proteins or marker proteins. The distribution of marker proteins in a phylogenetic tree shows evolutionary relationships within archaea. The functions of most of them are unknown although sequence comparisons indicate possible functions that still need to be proven.[39] Within the genome of some of the archaea, there is a conserved cluster of methanogenesis marker genes. (Table 2)

Table 2) The *mmp* cluster in some sequenced archaeal strains[39]

Name	Mm cluster (all genes in same direction) (NCBI)
Methanosphaera stadtmanae	*mmp2 -mmp3-mmp6-mmp5-mmp15-mmp17*
Methanothermobacter marburgensis	*mmp2 -mmp3-mmp6-mmp5-mmp15-mmp17*
Methanococcus maripaludis S2	no mm cluster
Methanospirillum hungate	*atwA -mmp3-mmp6-mmp5-mmp15-mmp17-mmp7*
Methanococcoides burtonii	*atwA -mmp3-mmp6-mmp5-mmp15-mmp17-mmp7*
Methanosarcina acetivorans	*atwA -mmp3-mmp6-mmp5-mmp15-mmp17-mmp7*

For example, in *M. acetivorans* C2A, the cluster contains *atwA*, *mmp3*, *mmp6*, *mmp5*, *mmp15*, *mmp17*, and *mmp7* genes, respectively. In *Methanosphaera stadtmanae*, *mmp7* and *atwA* are absent but *mmp2* is available in the cluster. Sometimes methanogenesis markers are spread out

or appear to be absent. There are no published papers or crystal structures on any of these methanogenesis marker proteins. But some of them contain a conserved protein domain.

Mmp3 contains the conserved protein domain of the cyclophilin family. The cyclophilin family catalyzes the cis-trans isomerization of the peptide bonds of proline residues. Chaperone functions have been reported for them too. They play an important role in protein folding or physiologically important processes such as opening a pore of a channel control with the molecular switch of the cis/trans isomerization of the peptide bond.[40] Mmp15 belongs to the nucleotide-binding domain (NBD) of the sugar kinase/HSP70/actin superfamily. HSP70 family proteins have a chaperone function. They have a nucleotide-binding domain (NBD) and a substrate-binding domain (SBD). The NBD binds ADP and ATP. The SBD binds a substrate. There is an allosteric communication between the two domains.[41–43]

1.1.11) Mcr Purification in the active form

Mcr enzyme can be purified in the active form when cells were pre-incubated with hydrogen gas. With this method, the specific activity of the cell extract was increased to about 2 μmol min^{-1} mg $protein^{-1}$ and after 10-fold purification, the specific activity was about 20 μmol min^{-1} mg $protein^{-1}$. In 1990, it was discovered that the preincubation of cells with 80% N_2 and 20% CO_2 leads to the Mcr_{ox1} state in the cell extract.[44]

Mcr_{ox1} can be converted to Mcr_{red1} using Ti(III) citrate as a reductant. Although Ti(III) citrate can also reduce Ni(II) to Ni(I) at alkaline pH, the reduction cannot happen with the Mcr enzyme silent.[45] The Mcr_{red1} form was estimated to have a specific activity of up to 100 μmol min^{-1} mg $protein^{-1}$.[21]

Mcr_{ox1} can be induced when cells are preincubated with sodium sulfide before harvesting.[46] The Mcr_{ox1} was subsequently converted by reduction with Ti(III) citrate to Mcr_{red1} with the same specific activity.

1.1.12) Attempts for activating Mcr with light

Wolfe and coworkers discovered that partial activation of the Mcr enzyme is possible when Mcr, HS-CoM, CH_3-S-CoM, and titanium (III) citrate are exposed to above 400 nm light. The products are the same as normal Mcr reactions. HS-CoM and CH_3-S-CoM cannot absorb the light but both titanium (III) citrate and Mcr can absorb the light. They did notice a color change in titanium (III) citrate is necessary for this reaction. The activation with light could not happen for the Ni in F_{430} free of the enzyme.[47] The authors proposed that the light induces a conformation change in the enzyme to be able to accept an electron from the reductant.

EPR signal of the active form of enzyme changes from Mcr_{red1} to Mcr_{red2} when the enzyme is incubated with coenzyme M and coenzyme B. Mcr_{red2} is a combination of axial Mcr_{red2a} spectrum and the rhombic MCR_{red2r} spectrum. Mcr_{red2a} is assigned to Ni(III) hydride complex. Mcr_{red2r} is assigned to a Ni(I)-S coordination.[21] The Mcr_{red2} EPR signal is light-sensitive. After 3 min of visible light radiation at 77 K, two new EPR signals appear. After 135 min of visible light radiation, one of those new EPR signals disappear and a third EPR signal shows. EPR signal of Mcr_{red2} completely disappeared after 195 min of irradiation. Mcr_{red2} EPR signal does not regenerate when light radiation stops. The process that occurs during light radiation to the Mcr_{red2} is not reversible.[48] (Figure 14) Another Mcr signal that is light-sensitive is the Mcr_{ox2} signal. This signal is induced when Mcr_{red1} is directly exposed to oxygen.[36,49]

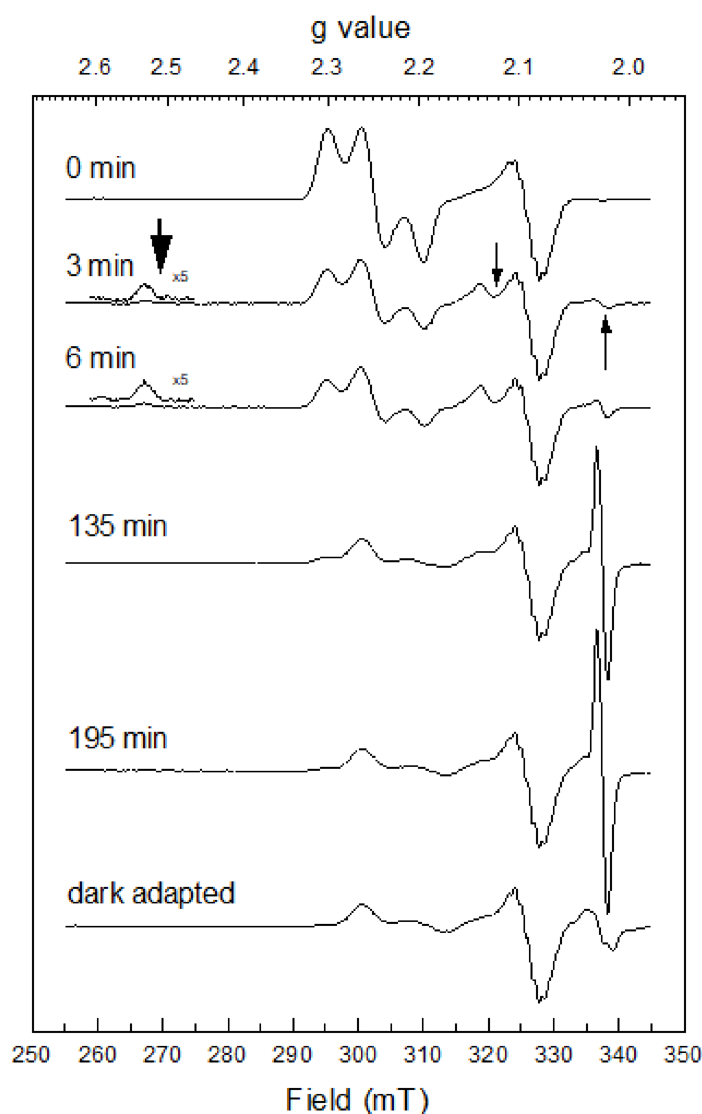

Figure 14) Mcr_{red2} EPR signal is light sensitive. A sample of Mcr_{red2} was exposed to a visible light. After 3 min of visible light radiation at 77 K, two new EPR signals appear. After 135 min of visible light radiation, one of those new EPR signals disappear and a third EPR signal shows. After 195 min of irradiation, Mcr_{red2} EPR signal disappeared and it does not regenerate when light radiation stops. The process that occurs during light radiation to the Mcr_{red2} is not reversible.[48] Taken with permission from (Mahlert et al. 2002). Copyright Springer Nature Switzerland AG

1.1.13) Cellular components responsible for activation of Mcr

Wolfe and coworkers were the first to purify Mcr. His group tried to purify Mcr and the component responsible for Mcr activation. The soluble part of the cell extract was separated into three activity-related components by DEAE-cellulose ion-exchange chromatography and named components A, B, and C based on their order of elution.[50] Component C is the Mcr enzyme and component B is HS-CoB.[50,51] Component A shows hydrogenase activity and was separated into three protein fractions A1, A2, and A3. Component A1 can be replaced with titanium (III) citrate as an electron donor and it is an F420-reducing hydrogenase.[52] A3 was isolated into two components A3a and A3b. A3a is a large size oxygen-sensitive brown complex (~500,000 Dalton) with one FeS center per 5,000 Daltons of A3a. A3a can be inhibited with ATP analogs, and it probably has an ATP hydrolysis site. It was proposed that the whole A3a complex works as an electron transfer system.[53] When Mcr was mixed with component A2, A3a, ATP, CH_3-S-CoM, HS-CoB, and Ti(III) citrate and incubated for a whole hour at 65 °C a specific activity of 0.1 μmol min^{-1} mg $protein^{-1}$ was achieved.[52,53]

1.1.14) Component A2

Component A2 has a molecular weight of around 60 kDa and is colorless and oxygen resistant. It can be purified with ATP-agarose affinity resin. Wolfe and coworkers did not report any ATP hydrolysis activity for A2 but Divya Prakash in the Duin lab showed that recombinant A2 protein expressed and purified from an *E. coli* host showed ATP hydrolysis activity (unpublished results). It cannot directly reduce NAD, NADP, FMN, and cofactor F_{430}. A2 has two ATP binding domains, one close to the N-terminal and one close to the carbon terminal of the protein. ATP binding domains of A2 are homolog to those of the ATP-binding cassette (ABC) superfamily of transport systems.[54] The ATP-binding cassette (ABC) superfamily of transport systems is responsible for the transportation of components across cell membranes such as drugs and organic components. They have four domain proteins, two of which are very hydrophobic and located in the cell membrane. The other two domains are located on the top of the hydrophobic domain toward the cytoplasmic membrane. These two domains have ATP binding sites, and they supply the energy for transportation. The substrate binds to the protein and triggers ATP binding. ATP binding free energy leads to conformational changes and the formation of the activated state. Upon substrate transport across the cell membrane, ATP hydrolysis to ADP and P_i forms a relaxed state of the protein.[54,55] Other proteins in this superfamily have different functions but all use ATP to steer certain processes.

1.1.15) Component A3a

To get a handle on the activation of Mcr, the work by Wolfe and coworkers was continued in the Duin lab after Ralph Wolfe retired. Component A3a was obtained relatively pure and the individual bands from SDS-PAGE were sent for identification with mass spectrometry. The components that were identified are shown in Table 3. The results show A3a is a multienzyme complex and contains F_{420}-reducing hydrogenase (Frh), F_{420}- nonreducing hydrogenase (Mvh), polyferredoxin, heterodisulfide reductase (Hdr), tungsten-containing formylmethanofuran dehydrogenase (Fwd) & molybdenum containing formylmethanofuran dehydrogenase (Fmd), methyl-coenzyme M reductase operon protein C (McrC), methanogenesis marker protein 7 (Mmp7), acetyl-coenzyme A (CoA) decarbonylase/synthase (Cdh), 5,10-methylenetetrahydromethanopterin reductase (Mer), and Fe-protein homolog/iron-sulfur cluster carrier protein/ATP-binding protein (SDP).

Table 3) Component A3a

1	F420-reducing hydrogenase (Frh)
2	F420- nonreducing hydrogenase (Mvh)
3	Heterodisulfide reductase (Hdr)
4	Tungsten-containing formylmethanofuran dehydrogenase (Fwd)
5	Molybdenum containing formylmethanofuran dehydrogenase (Fmd)
6	Methyl coenzyme M reductase operon protein C (McrC)
7	Methanogenesis marker protein 7 (Mmp7)
8	Acetyl-coenzyme A (CoA) decarbonylase/synthase (Cdh)
9	5,10-methylenetetrahydromethanopterin reductase (Mer)
10	ATP binding protein (SDP)
11	Iron-sulfur cluster protein (FFP)
12	UPF0145
13	Polyferredoxin

The protein was not completely pure but further purification attempts resulted in loss of activity. For some of these proteins, there does not appear to be a clear connection to Mcr activation and it had to be assumed they were not part of the complex. A model was proposed based on these results and assumptions (Figure 15)

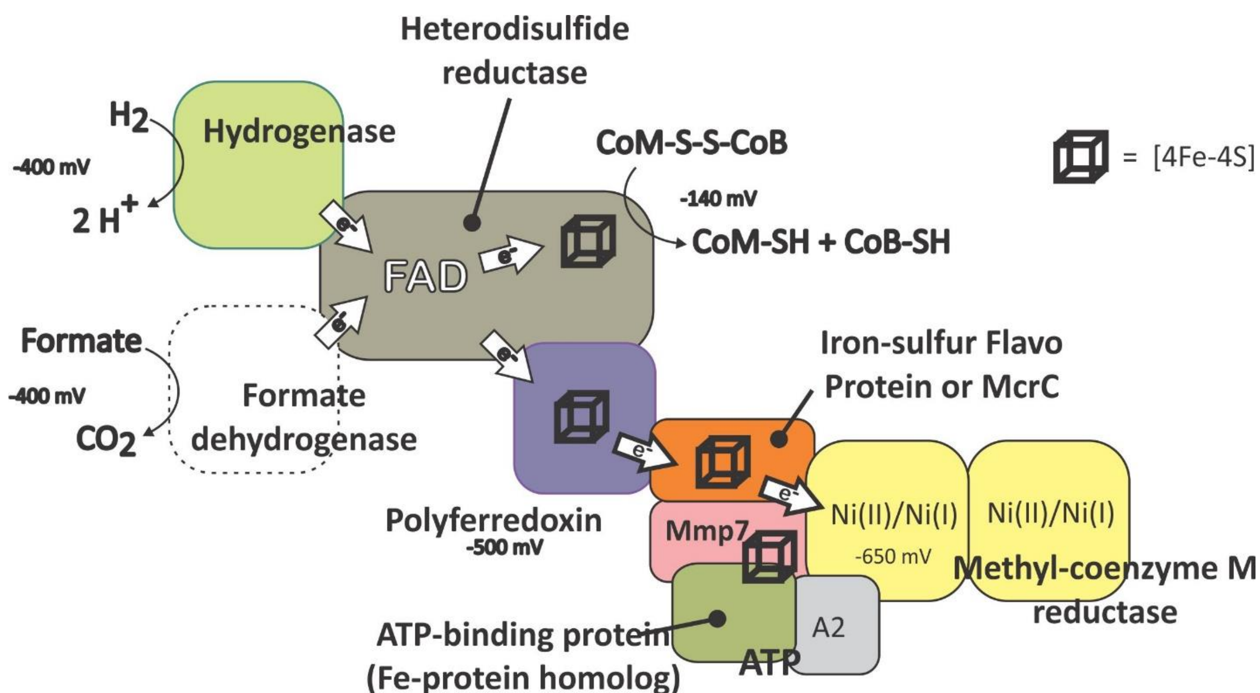

Figure 15) Proposed activation model for Mcr

The hydrogenases can provide the electrons for this process. When the organism is grown on formate the Fdh will play this role. Not shown here is Cdh which could play a role when CO is the electron source. The electrons are bifurcated at the FAD site and are used for heterodisulfide reduction at -200 mV and polyferredoxin reduction at -500 mV. This might not be enough for the Ni reduction in Mcr and ATP hydrolysis might be needed to further lower the potential similarly

to the Fe-protein in the activation of nitrogenases. The ATP binding enzyme is a Fe-protein homolog. The Cysteines that could potentially bind a bridged cluster, however, are placed at a different location in the A2 protein. In this work, we will show that A2, McrC, and Mmp7 all appear to bind clusters despite the absence of typical Cys motifs.[38]

Fe protein is a homodimer with C2 symmetry, and each subunit has an α/β domain. It has a 60 kDa molecular weight and is larger than other electron transfer proteins such as ferredoxins and flavodoxins. Fe protein has two ATP binding sites and one [4Fe-4S] cluster. The iron-sulfur cluster is located as a bridge between two subunits. If the cluster is removed the dimer structure is stable due to strong interactions between two subunits. The amino acid residues responsible for these interactions are the conserved sequence in most Fe proteins. In most ferredoxin proteins, an iron-sulfur cluster is buried within the protein, but the iron-sulfur cluster of Fe-protein is exposed to the solvent. According to the crystal structure, the distance between the iron-sulfur cluster and ATP binding site is around 20 Å. The binding of ATP changes the physicochemical properties of the cluster and leads to the lower redox potential of the cluster by about 100 mV. Upon electron transfer to the Fe_{ox}-$(ADP)_2$ protein, a reduced Fe protein forms, and an ADP is exchanged with ATP. Then, an activated complex of [Fe_{red} $(ATP)_2$; MoFe] forms with the utilization of ATP-free binding energy. An electron from Fe_{red} transfers to the MoFe protein. ATP hydrolyzes and results in dissociation of the tightly bound complex. Association and dissociation of the complex between Fe protein and MoFe protein are necessary for each electron-transfer step from Fe protein to the MoFe protein.[56–58]

1.1.16) Activation of Mcr with isolated A3a and recombinant A2

Duin and coworkers were able to activate the different forms for Mcr, ox1, and silent, using an assay that contained the recombinant A2, A3a, Mcr, MgATP, M-CoM, and dithiothreitol. 100% of Mcr_{ox1} and 65% of Mcr_{silent} was activated (Figure 16). The midpoint potential of dithiothreitol (E_m= 320 mV) is much higher than the midpoint potential of the Ni(II)/Ni(I) couple in the F_{430} bound enzyme (lower than -440 mV) or even higher than the midpoint potential of H_2/H^+ (E_m= 420 mV). The dithiothreitol was chosen to avoid direct activation by Ti(III)citrate

For the activation of Mcr, the reduction of Ni is necessary. For the reduction, it seems two steps are crucial. First, an electron needs to be transferred from an electron source to the Ni of the F_{430} bound enzyme. Maybe A3a works as an electron transfer system. Second, the electron needs to have enough potential to be able to reduce Ni of the F_{430} bound enzyme. An electron from electron sources such as H_2 or dithiothreitol has a higher midpoint potential and as a result, is not able to reduce Ni of F_{430} bound enzyme. Since activation of Mcr is ATP required process, utilization of ATP in component A2 or A3a may result in the formation of a low potential electron. Also, electron bifurcation can form a low potential electron. Utilization of both ATP and electron bifurcation may lead to making enough low potential electrons for the reduction of Ni in the enzyme.[38]

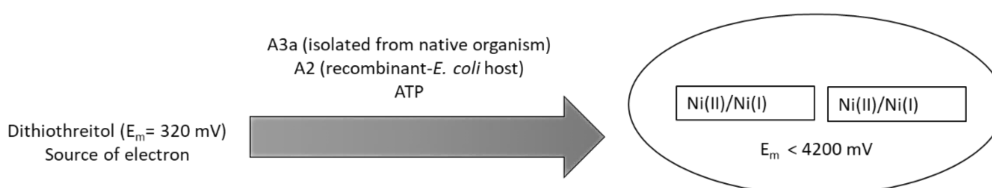

Figure 16) Activation of Mcrox1 and Mcr_{silent} with A3a and A2 components

Under the assumption that electrons are generated by hydrogenase and the Hdr complex can produce low potential electrons (-500mV), we proposed that a core complex containing McrC, Mmp7, component A2, ATP binding protein, and iron-sulfur flavoprotein (FFP) can function as a simplified activation complex using Ti(III)citrate as the electron source (Figure 17).

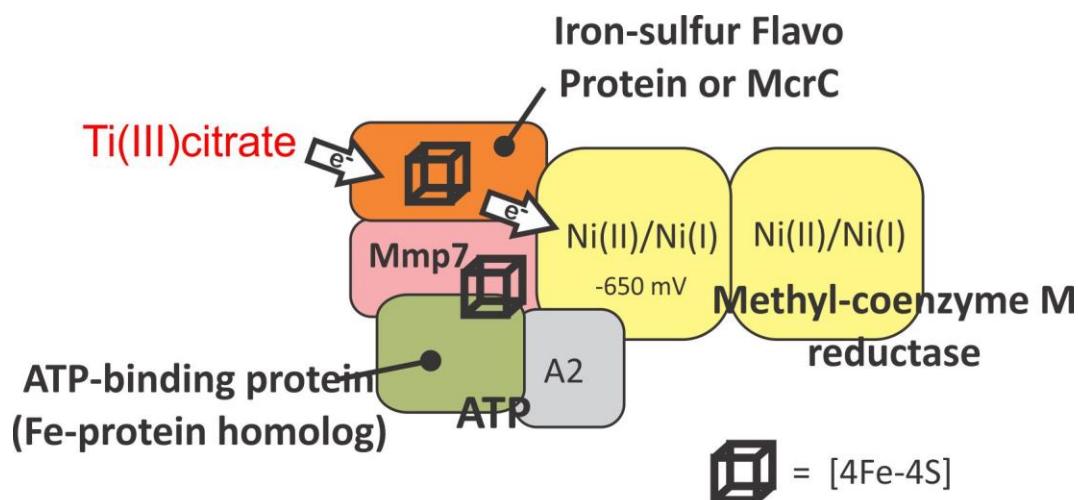

Figure 17) Proposed core activation complex responsible for activation of Mcr

1.1.17) First Attempts for activation of Mcr with heterologous expressed recombinant protein

For the first attempt, all five proteins in Figure 16 from *M. marburgensis* were cloned by Bryan Cronin and expressed in *E. coli,* and purified. Changes in the Mcr assay were monitored when all these five purified recombinant proteins were added to the Mcr. The result was disappointing since less than 1% activation was observed (unpublished results) similar to the experiments done by Wolfe and coworkers. There are several possible explanations. The first one is that recombinant archaeal proteins might not be produced properly in *E. coli*. A heterologous expression can result in the lack of the right chaperones, improper folding, lack of prosthetic group, and posttranslational modification in the recombinant protein. Even if the host is genetically close to the organism, there is a possibility the host cannot recognize the recombinant proteins. A homologous expression can avoid all possible mentioned problems. The second possible reason is there could be more than 5 components involved in the core activation complex. A pull-down experiment can help reveal any additional essential components. On the other side, using methanogenic archaea as a host is very new and there is not much information about it and the expression of recombinant protein in it. Growing this organism takes a long time. Preparation of the buffers and media is very time-consuming and expensive. The third possibility is that the used tag might interfere with the activation process. Since low cofactor content was observed the homologous expression might be the best option to get a properly working enzyme.

1.1.18) Megaplasmids in bacteria

The plasmids and vectors that are used have an interesting history. Results of genome sequencing of *Vibrio cholerae* reveal that each cell of *V. cholerae* had two circular chromosomes. One of them with a size of 2.96 Mb and the other one with a size of 1.07 Mb. It was found that the smaller chromosome replicates with a different mechanism from the large chromosome. The smaller chromosome has the same replication system of plasmids. Probably the smaller one accumulated the genes from external sources. The smaller chromosome is called a megaplasmid.[59]

A plasmid with *nod*, *nif*, and *fix* genes was found in *Rhizobium* and *Sinorhizobium* species of rhizobia bacteria and it was called a symbiotic plasmid. In *Bradyrhizobium* and *Mesorhizobium*, all three of the genes are located on the chromosome. *Sinorhizobium meliloti* 1021 contains two megaplasmids. One of them is 1.35 Mb and the other one is 1.68 Mb and they together form almost half of its 6.7 Mb genome. 12 plasmids are present in *Rhizobium leguminosarum biovar viciae* 3841 and represent about 40% of the 7.8 Mb genome. *Rhizobium etli* 42 has six plasmids representing one-third of the 6.5 Mb total genome.[60]

1.1.19) *Plasmid design*

Plasmids are circular double-stranded DNA. Three important parts of plasmid for cloning are replication, a selectable marker, and cloning sites. The origin of replication is about 1000 base pair regions named replicon and let plasmid replicate in the cells. Host bacteria provide the necessary enzymes for the replication of plasmids. Plasmids used for expression in methanogens are designed to replicate in one or two hosts. Plasmids are designed to encode antibiotic-resistance genes to mark and select the clones harboring the plasmids. Plasmids contain the cloning sites for the insertion of foreign DNA.[60]

Promoter sequences are short DNA sequences upstream of the genes where transcription machinery such as RNA polymerase binds. In this work, three promoters were used. Histone and phosphate regulated promoter for expression of recombinant protein in *M. maripaludis*. The phosphate-regulated promoter is found in archaea. Protein expression under the control of this promoter is regulated by the inorganic phosphate concentration of the media. When inorganic phosphate concentration (Pi) decease, recombinant gene expression is upregulated. But the growth of the archaea at low Pi is limited. Therefore, growth is separated from expression.[61] Histone promoter is found in *Methanococcus voltae*. Archaea do not have a nuclear membrane, but they have histones. Histone promoter is used for the expression of recombinant proteins in archaea. Histone genes are expressed during chromosome duplication in each cell cycle. And it is regulated based on the cell-cycle progression. Histone gene transcription and DNA replication work tightly together.[62,63] T7 promoter was used for the expression of recombinant protein in *E. coli*. T7 RNA polymerase bind to the T7 promoter and initiate the transcription. Protein expression of T7 RNA polymerase gene is under the control of lac promoter and it is regulated by Isopropyl β- d-1-thiogalactopyranoside (IPTG). IPTG works as an inducer and binds to the lac repressor and

stops inhibition of gene expression. As soon as T7 RNA polymerase is expressed, it initiates the expression of the recombinant protein under the control of T7 promoter. Protein expression of the recombinant protein is regulated by IPTG as an induced too. As soon as IPTG add to the *E. coli* culture, T7 RNA polymerase and recombinant proteins express.[64,65]

For replication of genomic DNA, initiator proteins detect specific sites on DNA and bind to them. This process results in the assembly of protein complexes with the role of activating the DNA helicases and unwinding DNA before replication. Subsequently, the replication machinery system loads and starts the replication. There is usually one replication origin in the genome of bacteria and archaea with small circular chromosomes. Eukaryotic genomes are very large, and they contain more replication origins. For example, there are about 400 origins in the genome of yeast. OriC is the replication origin of *E. coli* in a circular 4.6 Mb chromosome. OriC contains an A-T-rich DNA sequence which extremely facilitates the unwinding of DNA and 9-bp motifs for recognition and binding by the DnaA initial protein. Propagation of the plasmid replication is dependent on the host replication machinery. Host cells recognize the origin of the replication sequence of the plasmid and initiate the replication.[66]

1.1.20) Number of copies of a cloning plasmid vector

The number of copies of a plasmid available per chromosome is PCN. Plasmids can be low copies (1–10), medium copies (11–20), or high copies (>20). The origin of replication, and the type of host determine the PCN. Plasmid cloning vectors are usually unnecessary for the survival of the hosts. Replication of plasmids and expression of recombinant proteins needs a significant number of resources for the hosts and are considered selfish elements. Hosts prefer to eliminate the plasmids to save resources. PCNs are controlled by negative and positive regulatory systems which adjust the rate of replication of the plasmid. The product of the plasmids such as RNA or protein can have negative-regulating effects on replication. When plasmid concentration increases in the cell, the concentration of the negative regulator increases too. The rate of replication decreases, resulting in a negative feedback loop, which in turn stabilizes the copy number.[67]

1.1.21) Incompatibility among plasmids

The coexistence of different plasmids with similar replication systems in the same cell results in the loss of plasmids and those plasmids are called incompatible. The regulatory systems of the cell consider both plasmids identical and the control mechanism of copy number cannot differentiate between them. Eventually, after several replications, only one of the plasmids is kept by the cells and the other plasmid might be lost. Half of the cells have one plasmid and the other half have the second one.[59] (Figure 18)

Some plasmids with different replication origins designed for *E. coli* host cells are listed in table 4. pCDFDuet-1 origin is from Clo DF13 plasmid originated from *Enterobacter cloacae1*. The Clo DF13 plasmid is responsible for bacteriocins production. Some of the bacteria produce bacteriocins during their growth. Bacteriocins have antimicrobial activities to the other strains of the same species and they kill them.[68,69] pETDuet-1 origin is from ColE1 plasmids and they are

found in some bacteria producing colicin E1 such as Enterobacteriaceae. Plasmids with ColE1replicons are multicopy vectors. But on the other hand, the inserts are unstable if they are large and have repeated sequences. Replication of ColE1 plasmid starts from 555 bp upstream of the origin of replication. RNA polymerase transcript a pre-primer (RNAII). It hybridizes with the DNA template and forms a DNA-RNA hybrid. The hybrid has a secondary structure. Ribonuclease H (RNase H) is an endoribonuclease. RNase H can recognize the secondary structure of the DNA-RNA hybrid and cleaves it. A primer is produced and is used for DNA synthesis by DNA Polymerase I to replicate the plasmid. Transcription in the opposite direction of RNAII produces RNAI. The sequence of RNAII is complementary to the sequence of RNA I. They can bind together and make a stable RNA-RNA hybrid. RNAI is called antisense RNA and it works as an inhibitor. It is important to control the plasmid copy number.[70] pACYCDuet-1 origin is from plasmid pl5A and they are found in *E. coli* strain 15T. The size of the plasmid was about 2.2 kb.[71] pl5A, ColEl, CloDF13, and RSFlO30 plasmids have similar mechanisms for replication and regulation. P15A is compatible with ColEl, CloDF13, and RSF1030.[70] pRSFDuet-1 origin is from the plasmid RSF1030 which was discovered in *salmonella panama.* The plasmid size is 8.3 kb. The plasmid harbor the beta-lactamase gene which provides antibiotic-resistant.[72]

Table 4) Compatible plasmids with different replication origins for *E. coli* [73,69–72]

Plasmid	origin
pETDuet-1	ColE1 plasmids
pCDFDuet-1	CloDF13 plasmid
pACYCDuet-1	p15A origin
pRSFDuet-1	RSF1030

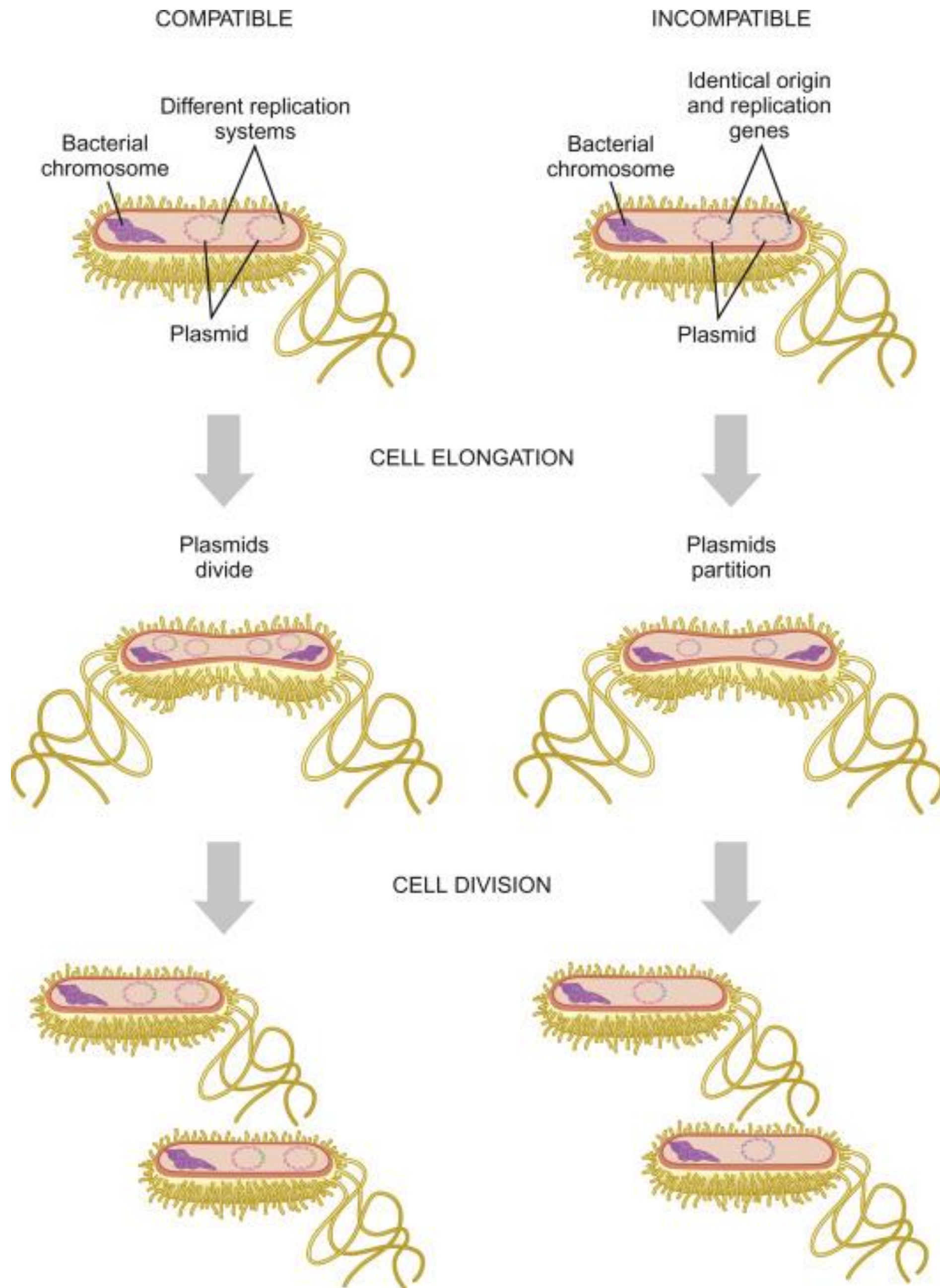

Figure 18) Compatible plasmids contain different origins of replication and can co-exist in the same bacterial cell and during cell division each daughter cell receive both plasmids. The incompatible plasmid contains identical origins of replication and after several cycles of division, each daughter inherits only one plasmid.[59] (Figure was adapted from Clark, Pazdernik, and McGehee 2019 with permission from Copyright © 2019 Elsevier.

1.1.22) C41(DE3) and BL21(DE3)pLysS competent cells

BL21 cells can only be used as a host for the expression of recombinant proteins under the control of promoters such as lac and T5 which are recognizable by the *E. coli* RNA polymerase. BL21(DE3) cells are encoded for T7 RNA polymerase under the control of the lacUV5 promoter in the chromosome which is inducible with IPTG.[74] T7 RNA polymerase is a small polymerase derived from bacteriophage T7 which is a virus for bacteria.[75] T7 RNA polymerase is about 7 times faster than *E. coli* RNA polymerase and it recognizes the *T7* promoter specifically. BL21(DE3) cells are suitable for the expression of recombinant protein under the control of the *T7* promoter. Expression of recombinant protein under control of *T7* promoter cannot happened with *E. coli* RNA polymerase. When expression of recombinant proteins is toxic to BL21(DE3) cells it can result in accumulation of expressed protein in inclusion body or low growth rate. C41(DE3) cells were derived from BL21(DE3) cells since they could express toxic and membrane recombinant proteins. Sequencing the complete genome of C41(DE3) showed that there are some mutations in the genome of C41(DE3) cells. These mutations weekend the lacUV5 promoter and lower the expression of T7 RNA polymerase which lead to different phenotype of the cells.[74]

Sometimes toxicity can still show up due to leaky expression. T7 lysozyme (T7Lys) is the inhibitor for T7 RNA polymerase. Inhibition of T7 RNA polymerase lower its activity. Adjustment of the activity of T7 RNA polymerase diminishes the leaky expression. *E. coli* BL21(DE3) pLysS contains a plasmid harboring a gene of T7Lys. Expression of the gene is induced by L-rhamnose. *E. coli* BL21(DE3) pLysS allows for optimization of protein expression of soluble proteins.[76] (Figure 19)

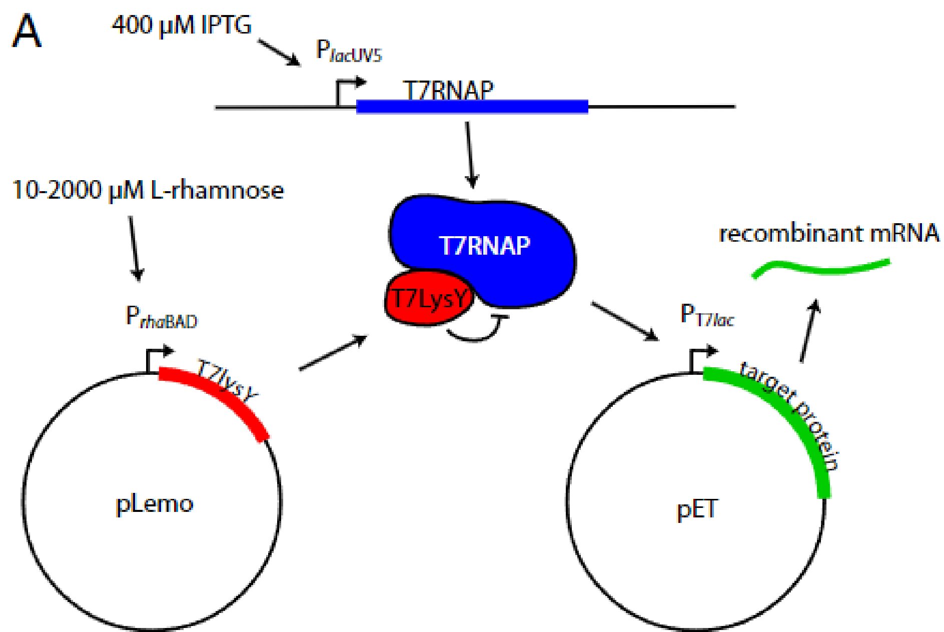

Figure 19) Expression of recombinant proteins in *E. coli* BL21(DE3) pLysS hosts. T7 RNA polymerase activity is adjusted with T7Lys inhibitor and decreases the leaky expression. T7Lys gene is cloned in pLemo plasmid and expressed under the control of *haBAD* promoter. Expression of the gene is induced by L-rhamnose.[76] Reproduced with permission from S. Wagner et al. 2008. "Copyright 2008 National Academy of Sciences"

1.1.23) MALDI-TOF fingerprinting mass spectroscopy

MALDI-TOF fingerprinting produces repeatable and reproducible results. It can be used to identify proteins from organisms from which the genome is known. [77] When proteins are digested with a specific protease, it results in a set of peptides (map) for each protein. The masses of these peptides are compared with a database that contains all the hypothetical masses for all the possible peptides, specific to that protease. In principle, the experiments can be performed with a mixture of proteins or subunits. There might be more than one mass match in the database for individual subunits making this method less straightforward. When the protein or subunit mixture is first applied to an SDS-PAGE, the individual band can be cut and analyzed by MALDI. In this case, fewer false positives will be present making the identification more straightforward.[78]

1.1.24) Outline of the Project

A. **Identification of A3a components**

A3a and A2 components together can activate Mcr. A3a is a large protein complex. Although the initial work in Dr. Duin's laboratory on the A3a protein complex was successful, there are still questions about the exact protein composition of A3a. When the A3a preparation was run on a native gel, 3 bands were obtained (unpublished results). It is possible that the A3a component was not pure. The four-column purification procedure was repeated multiple times, each time giving different results in composition and activating properties. Components might be lost in these long purification procedures. To get a better idea of what components are essential the 5 core A3a protein identified and also A2 will each be homologously expressed in *M. maripaludis.* It is expected that they will associate with the other components in the cell and that these will copurify. Through these 'pull-down' experiments we expect to find possible missing components. This method will be repeated with the newly found components until no new protein is detected anymore. All components will be characterized and checked for the presence of cofactors.

B. **Testing whether the *mmp* cluster encodes a large complex**

As an alternative approach, gene clusters of Mmp proteins will be studied. Methanogenesis marker proteins are unique to methanogenic archaea. There is a conserved cluster of methanogenesis marker genes in some methanogens. Mmp cluster proteins are regularly found associated with the A3a component (See section 1.1.15 for more details). It can be hypothesized that they are important for the activity or assembly of the Mcr enzyme. It is also not clear what the

function would be. The goal of this research is to check if the *mmp* cluster codes for a large complex and assemble a heteromeric protein complex.

C. Investigation of the function of Mmp cluster proteins

Based on the results in the lab, it was assumed that Mmp cluster proteins have important for the activity or assembly of Mcr. Co-expression of *mmp* cluster proteins and *mcr* operon genes could result in the assembly of McrA, McrB, and McrG and the production of Mcr enzyme. If they are involved in the activation, some protein-protein interactions could be detected between them and guide us to take the next step to investigate the function of *mmp* cluster gene.

Chapter 2 : Methanogenesis marker protein (Mmp) cluster

2.1) Introduction

Methanogens are anaerobic archaea, and they use anaerobic respiration to produce ATP. They use CO_2, methanol, methylamines, and acetate as substrates and produce a methyl-coenzyme M molecule from any of the used substrates and reduce it to methane. Mcr catalyzes the last step of anaerobic methane production in methanogenic archaea from two substrates, methyl-coenzyme M (CH_3-S-CoM) and coenzyme B (HS-CoM), producing methane and a heterodisulfide (CoB-S-S-CoM). Methanogenic archaea produce a significant amount of methane, and they have important roles in the carbon cycle and global climate.[5,6] Methane is a greenhouse gas and has a warming effect on the atmosphere temperature with one of the highest percentages of radiative forcing. Humans have an unquestionable role in increasing methane production by methanogens. Methanogens live in the ruminants of animals from livestock and produce a considerable amount of methane.[7,8]

On the other side, methane is a renewable energy source and can be used as a replacement for fossil fuels. It can be used to produce electricity, heat, and power for transportation.[14] Anaerobic digestion of waste and animal manure has been done since 1929 to break down complex organic compounds in wastewater with help of methanogens. In the first step, amino acids and fatty acids, and sugar are produced in the first fermentation step. In the second step, they break down into smaller molecules such as acetic acid and alcohol. Acetogenesis uses acetate to produce H_2 and CO_2. Methanogenesis uses CO_2 and H_2 to produce methane. Biogas is captured as fuel and used for energy.[79] (Figure 20) Because of the mentioned reasons, investigation of methanogens and Mcr are necessary.[14]

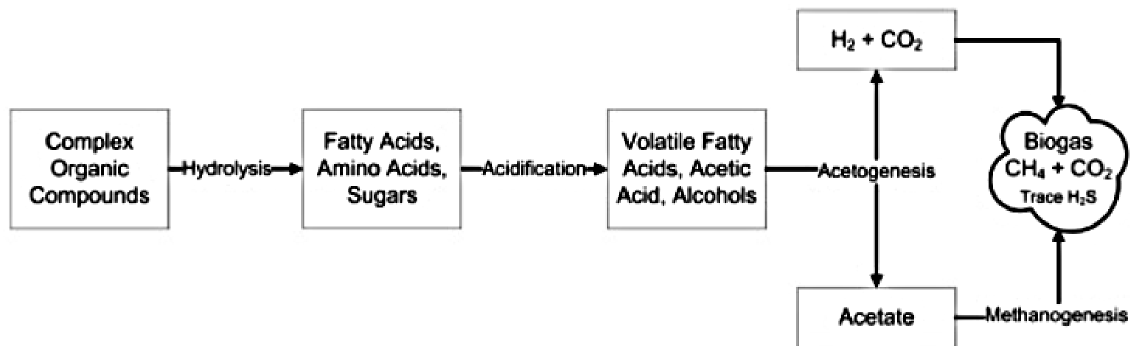

Figure 20) Anaerobic digestion of waste and animal manure leads to the breakdown of complex organic compounds. Amino acids, fatty acids, and sugar are produced in the first fermentation step. In the second step, they break down into smaller molecules such as acetic acids and alcohol. Acetogenesis uses acetate to produce H_2 and CO_2. Methanogenesis uses CO_2 and H_2 to produce methane. Biogas is captured as fuel and used for energy.[79] Republished with permission from McNab Stewart 2021. Copyright © 2022 Elsevier Ltd

Although the importance of the study of methanogens and Mcr is recognized by the researcher, progress is very slow. Methanogens are strict anaerobes, and any trace of oxygen can kill them. Growing them needs advanced facilities such as an anaerobic glovebox, apparatus for supplying oxygen-free gas, specialized glassware, fermenter, and culturing equipment.[14] All the buffers need to be oxygen-free. The preparation of media for methanogens is very time-consuming. Some of the methanogens grow very slowly. The amount of harvested wet cells is very low.

Recombinant protein expression is used to study and investigate proteins for more than three decades. Purification of proteins from natural sources is very time-consuming. And only a little amount of protein of interest is obtained. Protein production by recombinant protein expression made a revolution in protein investigation. *E. coli* cells have been used as a host for the

expression of recombinant proteins for more than three decades. They grow fast. Their glucose-salts media is cheap and easy to prepare for researchers. There are lots of publications about *E. coli* as a host and a wealth of knowledge is available about them. Lots of different expression plasmids and lots of engineered strains lead to a high level of expression of recombinant protein.[14] But sometimes there are challenges in the expression of archaea in *E. coli* cells host. Low-level expression of archaeal recombinant proteins is often observed due to different archaea codon usage of the mRNA from that of the *E. coli* host. Sometimes, archaea recombinant proteins expressed in *E. coli* misfolds or do not fold at all due to a lack of a native chaperon. The product is inactive and can aggregate in the inclusion bodies.[80,81]

Some specific proteins are unique to the archaea. They are named signature proteins or marker proteins. The functions of most of them, however, are unknown. Within the genome of some of the archaea, there is a conserved cluster of methanogenesis marker genes (Table 1).[39] In the prokaryotic genome, genes are not usually distributed randomly, and they are organized. Clusters of genes usually are functionally related in the same biological pathway.[80] Mmp cluster proteins are regularly found in the isolated A3a component. It can be assumed that Mmp cluster proteins could be important for the activity of Mcr or the assembly of Mcr. The *mmp* cluster in *M. acetivorans* contains *mmp7- atwA(A2)-mmp5-mmp15-mmp6-mmp17-mmp3* genes (Table 5). In the genome of some archaea, the *atwA* and *mmp7* genes are absent from the *mmp* cluster but *mmp2* is present. The *mmp2* in *M*. *acetivorans* is next to a 4Fe-4S cluster binding protein and *nifB*. NifB is a radical S-adenosyl-L-methionine (SAM) enzyme.[82] It is very important for nitrogenase cofactor assembly (Figure 21).

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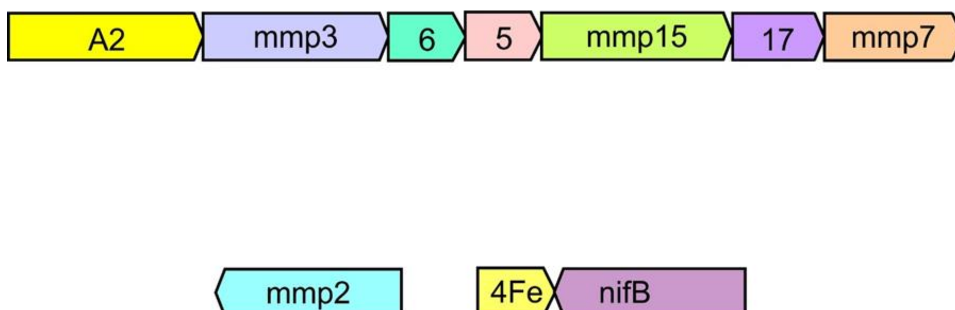

Figure 21) The *mmp* cluster and *mmp2* in *M. acetivorans* (NCBI)

Table 5) The *mmp* cluster from *M. acetivorans*

No	Name of the gene	Length of gene	Old locus tag	NCBI Reference Sequence	(kDa)
1	methyl coenzyme M reductase system, component A2 (*atwA*)	1614 nt	MA3998	WP_011023893.1	59.64
2	Methanogenesis marker 7 (*mmp7*)	936 nt	MA3992	WP_011023887.1	34.36
3	Methanogenesis marker 3 (*mmp3*)	1,578 nt	MA3997	WP_011023892.1	58.34
4	Methanogenesis marker 6 (*mmp6*)	444 nt	MA3996	WP_048065869.1	17.83
5	Methanogenesis marker 5 (*mmp5*)	504 nt	MA3995	WP_048066559.1	18.67
6	Methanogenesis marker 15 (*mmp15*)	1,248 nt	MA3994	WP_011023889.1	44.02
7	Methanogenesis marker 17 (*mmp17*)	600 nt	MA3993	WP_011023888.1	22.75
8	Methanogenesis marker 2 (*mmp2*)	999 nt	MA4193	WP_011024078.1	35.59

The first research goal is the expression of the *mmp* cluster genes in *E. coli* and to check if they encode for a large complex. If they do, it means they are functionally related in the same biological pathway. Detection of prosthetic groups in proteins and the study of protein-protein interactions between the Mmp cluster proteins can lead us to investigate if they work together and what can be a possible function. For this research, the *mmp* cluster from *M. acetivorans* was cloned

with no tag for the *E. coli* system. Each gene was cloned with a separate promoter. The recombinant protein was purified with ATP resin. Based on this study, the *mmp* cluster encodes a large complex.

The second research goal is to check if *mmp* cluster genes are involved in the assembly or activation of Mcr. Co-expression of the *mmp* cluster and *mcr* operon genes could reveal the function of the Mmp cluster. The co-expression should lead to the proper assembly of McrA, McrB, and McrG and the production of the Mcr enzyme or could give some information about protein-protein interaction between the Mmp cluster and Mcr operon proteins. For this research, *mcr* operon was cloned from *M. acetivorans* and *McrA* was cloned with a histidine tag. A McrA gene with an optimized codon for expression in *E. coli* was used for cloning.

2.2) Materials

N6-(6-Amino)hexyl-ATP-Agarose (6AH-ATPAgarose) resin was purchased from Jena Bioscience. Phusion High-Fidelity DNA Polymerase was purchased from New England Biolabs. NEBuilder HiFi DNA Assembly Cloning Kit was purchased from New England Biolabs. 1.2% FlashGel DNA Cassette and FlashGel DNA marker (100 bp – 4 kb) were purchased from Lonza. Z.N.A Gel Extraction Kits was purchased from Omega. EconoTaq PLUS GREEN 2X Master Mix was purchased from Thermo Scientific. Nanosep Centrifugal Devices Membrane 10 and 3K were purchased from Omega. Protease Inhibitor Cocktail Tablets were purchased from Roche. Single antibody (histidine tag, Alkaline Phosphatase detection) was purchased from Sigma-Aldrich. Primary antibody (Histidine tag) and Secondary Antibody (Alkaline phosphatase) were purchased from BIO-RAD. Alkaline phosphatase (AP) color development was purchased from BIO-RAD. Primary Antibody (6x-His Tag) and Secondary Antibody with Horseradish Peroxidase detection (HRP) and HRP color development (Metal Enhanced DAB Substrate Kit) were purchased from Invitrogen.

2.3) Experiments

2.3.1) Cloning of mmp cluster

The pETDuet-1 plasmid was selected for expression of the 8 genes since it is a high copy number plasmid. Each gene was cloned with a separate promoter rather than a shared promoter. Studies showed that the expression of two target genes under one promoter results in low expression of the gene that is at a longer distance from the promoter.[83] To achieve this goal, four Duet plasmids were used. Duet plasmids are for the co-expression of two target genes with two promoters. A SwaI restriction site was mutated already in the upstream sequence of the promoter of the first cloning site of pCDFDuet and pACYCDuet by lab mates in Dr. Mansoorabadi's lab. The SwaI restriction site lets researchers avoid unnecessary PCR experiments and as a result, prevents any possible mutation. The Gibson assembly method and restriction enzyme method both were used to assemble all 8 genes in one plasmid.

The 8 gene plasmid was prepared in 4 steps, and they will be explained in detail in the next sections. None of the genes contain a tag because tags sometimes disrupt interactions of proteins and their function.[83] Seven plasmids were needed for the four-step process. Each of them was assigned a name and they can be found in table 6. The *mmp* cluster plasmid is the desired plasmid and contains all 8 genes.

Table 6) Seven plasmids were assembled during the cloning of 8 genes in a pETDuet-1 plasmid. Each of them was assigned a name.

Assembled plasmid	Given name
pETDuet-*mmp5-mmp15*	SH-1
pCDFDuet-SwaI-*mmp6-mmp17*	SH-2
pACYCDuet-SwaI-*mmp7- atwA*	SH-3
pETDuet-*mmp3-mmp2*	SH-4
pETDuet-SwaI-*mmp6-mmp17-mmp5-mmp15*	SH-5
pETDuet-SwaI-*mmp7- atwA -mmp6-mmp17-mmp5-mmp15*	SH-6
pETDuet-*mmp7-atwA-mmp3-mmp2-mmp6-mmp17-mmp5-mmp15*	*mmp* cluster plasmid

2.3.1.A) Cloning design

First step: Eight genes were cloned into four different plasmids creating pETDuet-*mmp5*-*mmp15* (SH-1), pCDFDuet-SwaI-*mmp6*-*mmp17* (SH-2), pACYCDuet-SwaI-*mmp7*- *atwA* (SH-3) and pETDuet-*mmp3*-*mmp2* (SH-4) (Table 7 and Figure 22).

Table 7) The first step of cloning the *mmp* cluster

Genes	Plasmids	Assembled plasmid	Given name
mmp5 and *mmp15*	pETDuet-1	pETDuet-*mmp5*-*mmp15*	SH-1
mmp6 and *mmp17*	pCDFDuet-SwaI-1	pCDFDuet-SwaI-*mmp6*-*mmp17*	SH-2
mmp7 and *atwA*	pACYCDuet-SwaI-1	pACYCDuet-SwaI-*mmp7*- *atwA*	SH-3
mmp3 and *mmp2*	pETDuet-1	pETDuet-*mmp3*-*mmp2*	SH-4

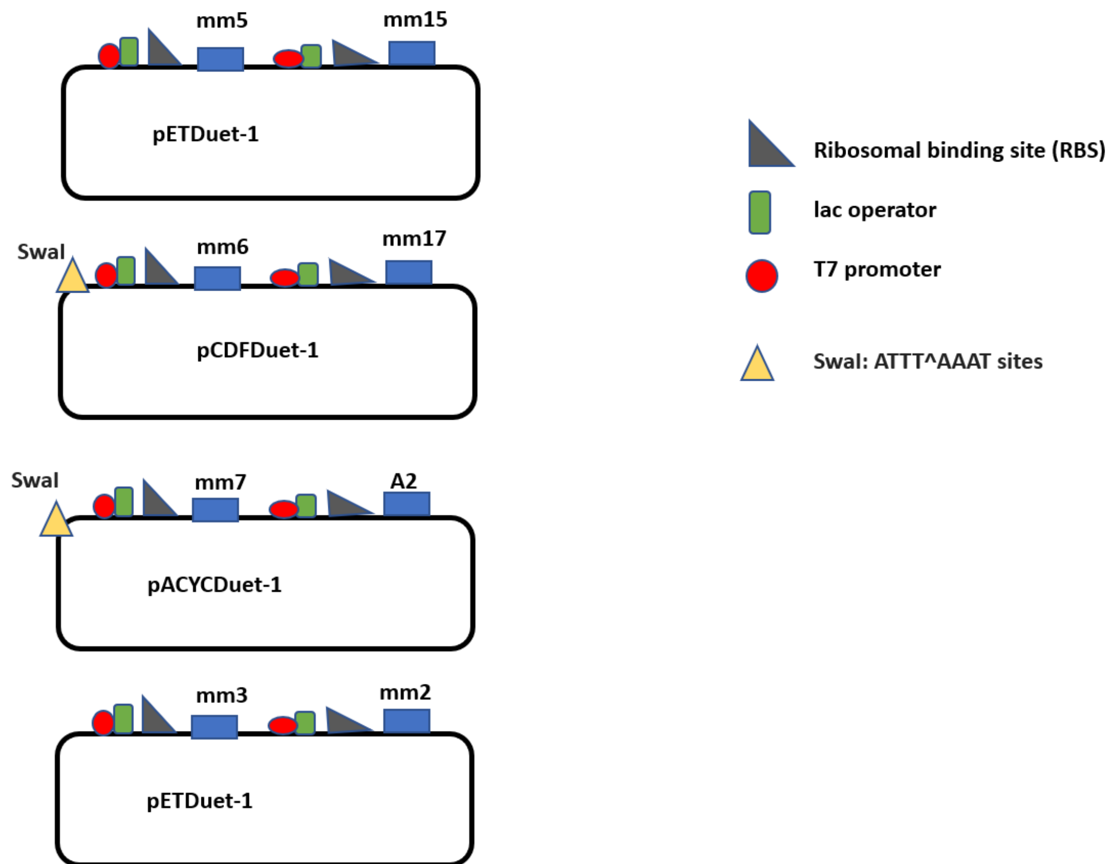

Figure 22) The first step for cloning of *mmp* cluster. pACYCDuet-SwaI-1 and pCDFDuet-SwaI-1 already have SwaI restriction sites mutated by lab mates in Dr. Mansoorabadi's lab. SwaI site is in the upstream sequence of the promoter of the first cloning site. Each gene contains a ribosome binding site, promoter, and lac operator.

Second step: pETDuet-SwaI-*mmp6-mmp17-mmp5-mmp15* (SH-5) was assembled from pETDuet-*mmp5-mmp15* (SH-1) and pCDFDuet-SwaI-*mmp6-mmp17* (SH-2) (Figure 23).

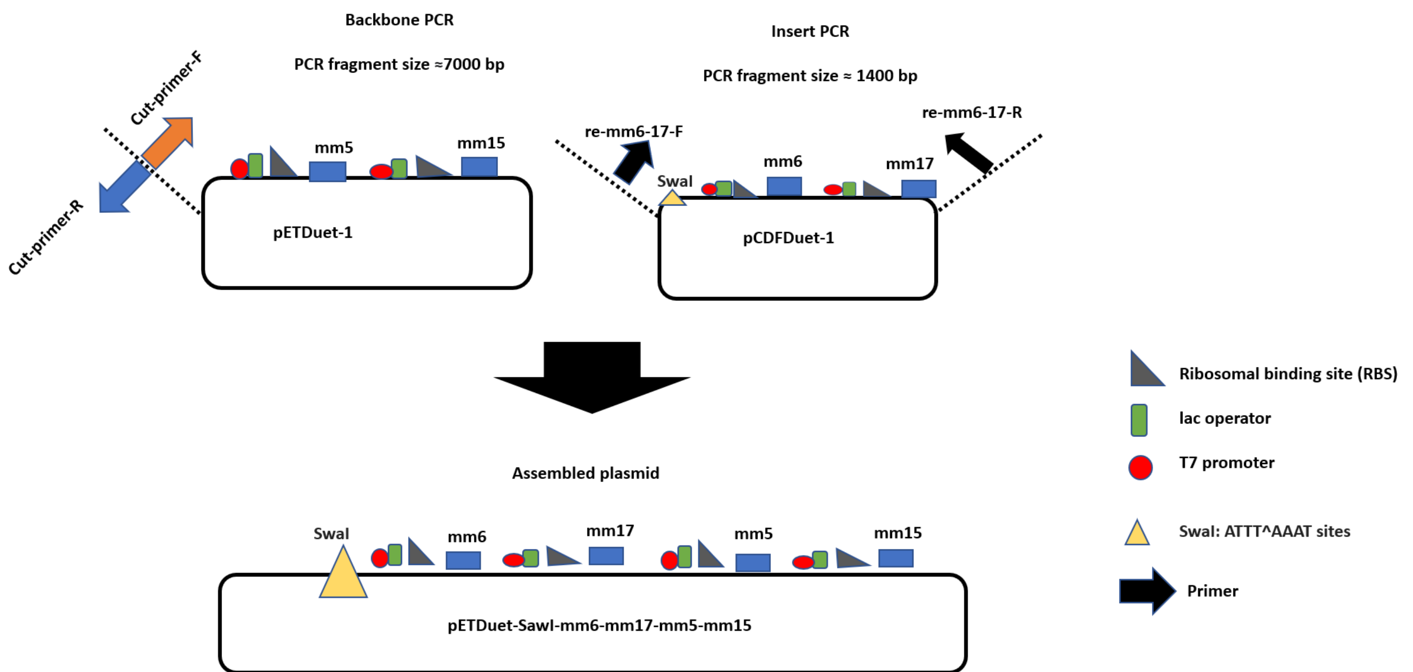

Figure 23) The second step for cloning the mm cluster. A PCR fragment (1400 bp) containing the *mmp6* gene and *mmp17* gene, their ribosome binding sites, lac operators and promoters, and the SwaI restriction site was inserted into pETDuet-*mmp5-mmp15*. pETDuet-*mmp5-mmp15* plasmid was linearized with PCR (cut-primer-F and cut-primer-R). The insertion of two new genes was upstream of the promoter of the *mmp5* gene.

Third step: pETDuet-SwaI-*mmp7- atwA -mmp6-mmp17-mmp5-mmp15* (SH-6) was made from pETDuet-SwaI-*mmp6-mmp17-mmp5-mmp15* (SH-5) and pACYCDuet-SwaI-*mmp7- atwA* (SH-3) (Figure 24).

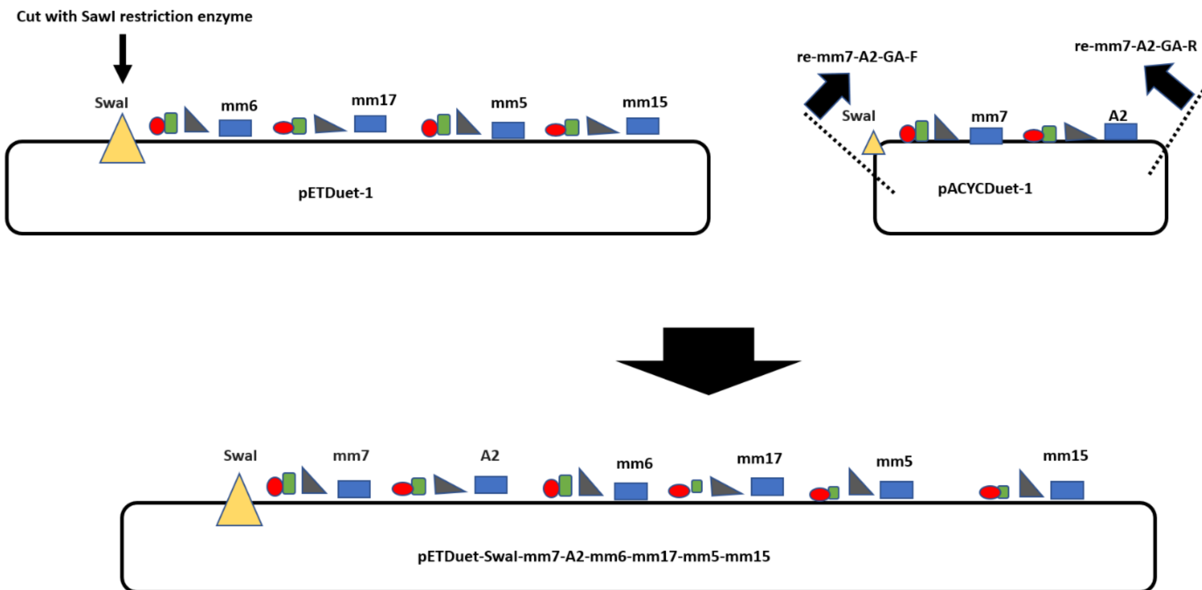

Figure 24) The third step for cloning the mm cluster. A PCR fragment containing the *Mmp7* gene and *A2* gene, their ribosome binding sites, lac operators and promoters, and the SwaI restriction site was inserted into pETDuet- *mmp6-mmp17- mmp5-mmp15*. pETDuet- *mmp6-mmp17- mmp5-mmp15* plasmid was linearized with the SwaI enzyme. The insertion of two new genes was upstream of the promoter of the *mmp6* gene. *atwA* defines *A2* gene

Final step: pETDuet-*mmp7- atwA-mmp3-mmp2-mmp6-mmp17-mmp5-mmp15* (*mmp* cluster plasmid) was assembled from pETDuet-SwaI-*mmp7- atwA -mmp6-mmp17-mmp5-mmp15* (SH-6) and pETDuet-*mmp3-mmp2* (SH-4) (Figure 25). All the genes were amplified from the chromosomal DNA of *Methanosarcina acetivorans* C2A.

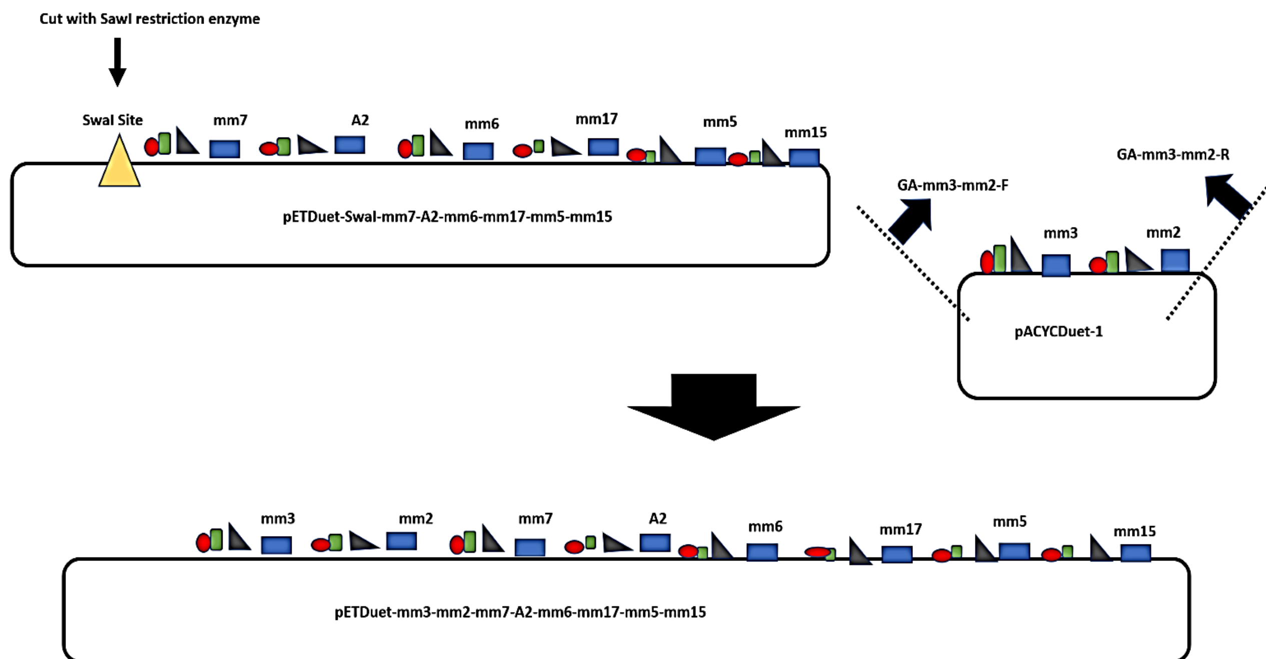

Figure 25) The fourth step of cloning the *mmp* cluster. A PCR fragment containing the *mmp3* gene and *mmp2* gene, their ribosome binding sites, lac operators and promoters, and SwaI restriction site was inserted into pETDuet- *mmp7- atwA - mmp6-mmp17- mmp5-mmp15*. pETDuet-*mmp6-mmp17 -mmp5-mmp15* plasmid was linearized with the SwaI enzyme. The insertion of two new genes was upstream of the promoter of the *mmp6* gene. *atwA* defines the *A2* gene.

2.3.1.B) Cloning procedure

For each assembled plasmid one name was assigned (Table 6). The procedure for cloning them is as below:

A. Cloning of plasmid SH-1

The *mmp15* gene (1248 bp) was amplified from the chromosomal DNA of *M. acetivorans* C2A with primers GA-MM15-FW and 5´- GA-MM15-RW to be inserted in the first cloning site of the pETDuet-1 plasmid. Phusion® High-Fidelity DNA Polymerase and HF buffer were used for the PCR. PCR samples were mixed with FlashGel™ loading dye and loaded into the tiers of the 1.2% FlashGel® DNA Cassette wells along with FlashGel™ DNA marker (100 bp – 4 kb) and the band with the correct size was isolated and DNA was extracted from the gel with E.Z.N.A.® Gel Extraction Kits and was eluted in nuclease-free water and stored at -20 °C. The linear backbone was amplified from the pETDuet-1 plasmid with RE-1-PETDUET-FW and RE-1-PETDUET-RW primers. Phusion® High-Fidelity DNA Polymerase and GC buffer were used for the PCR. PCR sample was run on a 1.2% FlashGel® DNA Cassette and the correct band was isolated, and the DNA fragment was extracted from the gel into nuclease-free water. The Gibson assembly mixture was prepared with the DNA insert fragment, DNA backbone fragment, nuclease-free water, DMSO (0.2 µL), and Gibson Assembly Master Mix (2×) (10 µL). The ratio of insert DNA (pmol) to backbone DNA (pmol) was around 6. The Gibson assembly mixture was heated for 30 min at 50 °C. The mixture was put on ice and then transformed into the NEB 5-alpha competent *E. coli*. Two colonies were selected and grown in a 5 mL autoclaved LB medium with 8 µl ampicillin (100 ng/mL). Plasmids of each culture were isolated. PCR experiments were carried out to verify the

successful insert of the *mmp15* gene in the assembled plasmid. The verified plasmid was sent for DNA sequencing. Sequences of primers can be found in Table 8.

To make the pETDuet-*mmp5*-*mmp15* plasmid, the *mmp5* gene (513 bp) was amplified from the chromosomal DNA of M. acetivorans C2A with primers GA-MM5-2-PETDUET-FW and GA-MM5-2-PETDUET-RW to be inserted into the second cloning site of the pETDuet-*mmp15*(CS1) plasmid. The linear backbone was amplified from the pETDuet- *mmp15*(CS1) plasmid with primers RE-2-PETDUET-FW and RE-2-PETDUET-RW. The Gibson assembly mixture was prepared. The ratio of the DNA insert (pmol) to backbone DNA (pmol) was around 3. The rest of the procedure was the same as the cloning of mmp15 in the first cloning site of pETDuet-1. Sequences of primers can be found in Table 8.

Table 8) Primers for cloning SH-1

Primers names	5′ to 3′
RE-1-PETDUET-FW	GGTATATCTCCTTCTTAAAGTTAA
RE-1-PETDUET-RW	TCGAACAGAAAGTAATCG
RE-2-PETDUET-FW	TCTACTAGCGCAGCTTAAT
RE-2-PETDUET-RW	ATGTATATCTCCTTCTTATACTTAACTAATA
PCR-MM15-FW	ATGAGTGCCGATGAA
PCR-MM15-RW	TCACTCCTCCACGTAG
PCR-MM5-FW	GTGAATTTTTTGGCAAAG
PCR-MM5-RW	TCATTTTTCACCCTCCTC
GA-MM15-1- PETDUET -FW	TTTAACTTTAAGAAGGAGATATACCATGAGTGCCGATGAA
GA-MM15-1- PETDUET -RW	CGATTACTTTCTGTTCGATCACTCCTCCACGTAG
GA-MM5-2-PETDUET-FW	TAAGTATAAGAAGGAGATATACATGTGAATTTTTTGGCAAAG
GA-MM5-2-PETDUET-RW	GGTTATGCTAGTTATTGCTCAGCGGTCATTTTTCACCCTCCTC

B. Cloning of plasmid SH-2

The *mmp6* gene (444 bp) was amplified from the chromosomal DNA of *M. acetivorans* C2A with primers GA-MM6-1-PCDF-FW and 5´- GA-MM6-1-PCDF-RW to be inserted in the first cloning site of the pCDFDuet-1 plasmid. The linear backbone was amplified from a pCDFDuet-1 plasmid with primers RE-1-PCDF-FW and RE-1-PCDF-RW. The Gibson assembly mixture was prepared. The ratio of insert DNA (pmol) to backbone DNA (pmol) was around 3. Sequences of primers can be found in Table 9.

To make the pCDFDuet-SwaI-*mmp6*-*mmp17*, the *mmp17* gene (600 bp) was amplified from chromosomal DNA of *M. acetivorans* C2A with primers GA-MM17-2-PCDF-FW and GA-MM17-2-PCDF-RW to be inserted in the second cloning site of the pCDFDuet-*mmp6* plasmid. The linear backbone was amplified from pCDFDuet-*mmp6* plasmid with primers RE-2-PCDF-RW and RE-2-PCDF-FW. Sequences of primers can be found in table 9.

Table 9) Primers for cloning SH-2

Primers names	5´ to 3´
RE-1-PCDF-FW	TCGAACAGAAAGTAATCG
RE-1-PCDF-RW	GGTATATCTCCTTATTAAAGTTAAAC
PCR-MM17-FW	ATGGATTCGCTTGAGAC
PCR-MM17-RW	TTACTTTTTCCCTCCTTCC
PCR-MM6-FW	ATGACAGAAATAAAACAAAATG
PCR-MM6-RW	TCACCTCGAAAGTTCC
GA-MM6-1-PCDF-FW	GTTTAACTTTAATAAGGAGATATACCATGACAGAAATAAAACAAAATG
GA-MM6-1-PCDF-RW	AATACGATTACTTTCTGTTCGATCACCTCGAAAGTTCC
GA-MM17-2-PCDF-FW	TTAAGTATAAGAAGGAGATATACATATGGATTCGCTTGAGAC
GA-MM17-2-PCDF-RW	GGTTATGCTAGTTATTGCTCAGCGGTTACTTTTTCCCTCCTTCC

C. Cloning of plasmid SH-3

The *mmp7* gene (936 bp) was amplified from the chromosomal DNA of *M. acetivorans* C2A with primers GA-MM7-1-PACYC-FW and GA-MM7-1-PACYC-RW to be inserted in the first cloning site of pACYCDuet-1 plasmid. The linear backbone was amplified from the pACYCDuet-1 plasmid with primers RE-1-PACYC-F and RE-1-PACYC-R. The Gibson assembly mixture was prepared with a ratio of 3. Sequences of primers can be found in table 10.

The *atwA* gene was cloned into the second cloning site of the pACYCDuet-*mmp7*(cs1). The gene was amplified from the chromosomal DNA of *M. acetivorans* C2A with primers GA-A2-2-PACYC-RW and GA-A2-2-PACYC-FW for insertion in the second cloning site of the pACYCDuet-*mmp7* plasmid. The linear backbone was amplified from the pACYCDuet-*mmp7* plasmid with RE-SEC-PACY-R and RE-SEC-PACY-F primers. The Gibson assembly mixture was prepared and the ratio between the DNA insert fragment (pmol) and DNA backbone fragment was around 3. Sequences of primers can be found in Table 10.

Table 10) Primers for plasmid SH-3

Primer names	5´ to 3´
RE-1- PACYC -FW	TCGAACAGAAAGTAATCG
RE-1- PACYC -RW	GGTATATCTCCTTATTAAAGTTAAAC
RE-2- PACYC -FW	TCTACTAGCGCAGCTTAAT
RE-2- PACYC -RW	ATGTATATCTCCTTCTTATACTTAACTAATA
PCR-MM7-FW	ATGGTTGTACTCCTACCCTAT
PCR-MM7-RW	TCAAATTGCAAAACCAAC
PCR-A2-FW	ATGCCAGTATTTATTGAAG
PCR-A2-RW	TTATATTTCTTCTGCAGCT
GA-MM7-1-PACYC-FW	GTTTAACTTTAATAAGGAGATATACCATGGTTGTACTCCTACCCTAT
GA-MM7-1-PACYC-RW	GTACAATACGATTACTTTCTGTTCGATCAAATTGCAAAACCAAC
GA-A2-2-PACYC-RW	TAGTTAAGTATAAGAAGGAGATATACATATGCCAGTATTTATTGAAG
GA-A2-2-PACYC-RW	AGCCTAGGTTAATTAAGCTGCGCTAGTAGATTATATTTCTTCTGCAGCT

D. Cloning of plasmid SH-4

pETDuet-*mmp3* (cs1) plasmid was already available in Dr. Mansoorabadi's lab. The *mmp2* gene (999 bp) was amplified from the chromosomal DNA of *M. acetivorans* C2A with primers named GA-MM2-F and GA-MM2-R to be inserted in the second cloning site of the pETDuet-*mmp3* plasmid. The linear backbone was amplified from pETDuet-*mmp3* with primers RE-SEC-PACYC-R and named RE-SEC-PACYC-F. The ratio of insert DNA (pmol) to backbone DNA (pmol) was around 3. Sequences of primers can be found in Table 11.

Table 11) Primers for plasmid SH-4

Primers names	5′ to 3′
RE-SEC-PACYC-R	ATGTATATCTCCTTCTTATACTTAACTAATA
RE-SEC-PACYC-F	TCTACTAGCGCAGCTTAAT
GA-MM2-F	ATATTAGTTAAGTATAAGAAGGAGATATACATATGAATCTGGAAGAGCTAGC
GA-MM2-R	ATTAAGCTGCGCTAGTAGATTATTCGGAACCTATACCTGTT

E. Cloning of plasmid SH-5

The insert (1325 bp) including *mmp6* and *mmp17* genes from the pCDFDuet-*mmp6-mmp17* plasmid (SH-2) was amplified with primers RE-MM6-17-F and RE-MM6-17-R. The linear backbone (6935 bp) was amplified from pETDuet- *mmp5-mmp15* plasmid (SH-1) with primers CUT-PETDUET-F and CUT-PETDUET-R. The Gibson assembly mixture was prepared. The ratio of the DNA insert (pmol) to backbone DNA (pmol) was around 5. For the verification of the successful insert of the fragment, the isolated plasmid and the pETDuet-*mmp5-mmp15* both were linearized with SwaI enzyme, and then their size was compared. In the next step, the PCR experiments were carried out, and the samples were sent for sequencing. Sequences of primers can be found in table 12. The design of the plasmid assembly can be found in Figure 23.

Table 12) Primers for plasmid SH-5

Primers names	5´ to 3´
RE-MM6-17-F	ATGCGTCCGGCGTAGAGGATCGAGGATCTCGACGCTCTCCCT
RE-MM6-17-R	TCGCGGGATCGAGATCGATCTGCTAGTTATTGCTCAGCGG
CUT-PETDUET-F	GATCGATCTCGATCCC
CUT-PETDUET-R	TCGATCCTCTACGCC

F. Cloning of plasmid SH-6

The insert (2865 bp) including *atwA* and *mmp7* genes from pACYCDuet-*mmp7-atwA* plasmid (SH-3) was amplified with primers RE-MM7-A2-GA-F and RE-MM7-A2-GA-R. The pETDuet -SwaI-*mmp6-mmp17- mmp5-mmp15* plasmid (SH-5) was linearized with SwaI enzyme and was purified with a 1.2% FlashGel DNA Cassette and the DNA fragment was extracted from the gel into the nuclease-free water. The Gibson assembly mixture was prepared. The ratio of insert DNA (pmol) to backbone DNA (pmol) was around 5. For the verification of the successful insert of the fragment, the assembled plasmids and plasmid SH-5 (template plasmid) were linearized with SwaI enzyme, and then their size was compared. In the next step, the PCR experiments were carried out, and then samples were sent for sequencing. Sequences of primers can be found in Table 13. The design of the plasmid assembly can be found in Figure 24.

Table 13) Primers for plasmid SH-6

Primers names	5′ to 3′
RE-MM7-A2-GA-F	AGGATCTCGACGCTCTCCCTTatttGGATCTCGACGCTCTCC
RE-MM7-A2-GA-F	GTATTAATTTCCTAATGCAGGAtttGCTAGTTATTGCTCAGCGG

G. Cloning of plasmid *mmp* cluster plasmid

The insert (2961 bp) including *mmp3* and *mmp2* genes from the pETDuet-*mmp3-mmp2* plasmid (SH-4) was amplified with primers GA-MM3-MM2-R and GA-MM3-MM2-F. Phusion High-Fidelity DNA Polymerase and GC buffer were used for the PCR For the backbone DNA fragment, the pETDuet *component A2-mmp7-mmp6-mmp17- mmp5-mmp15* plasmid (SH-6) was linearized with SwaI enzyme and purified on a 1.2% FlashGel DNA Cassette. The Gibson assembly mixture was prepared. The ratio of insert DNA (pmol) to backbone DNA (pmol) was around 10. For the verification of the successful insert of the fragment, the assembled plasmids and template plasmid for cloning SH-6 were linearized with the SwaI enzyme, and then their size was compared. The samples were sent for sequencing. Sequences of primers can be found in Table 14. The design of the plasmid assembly can be found in Figure 25. The map is shown in Figure 26.

Table 14) Primers for plasmid *mmp* cluster plasmid

Primers names	5´ to 3´
GA-MM3-MM2-R	GTATTAATTTCCTAATGCAGGATTTTGCTAGTTATTGCTCAGCGG
GA-MM3-MM2-F	TGGATCTCGACGCTCTCCCTTATTTATGCGTCCGGCGTAGA

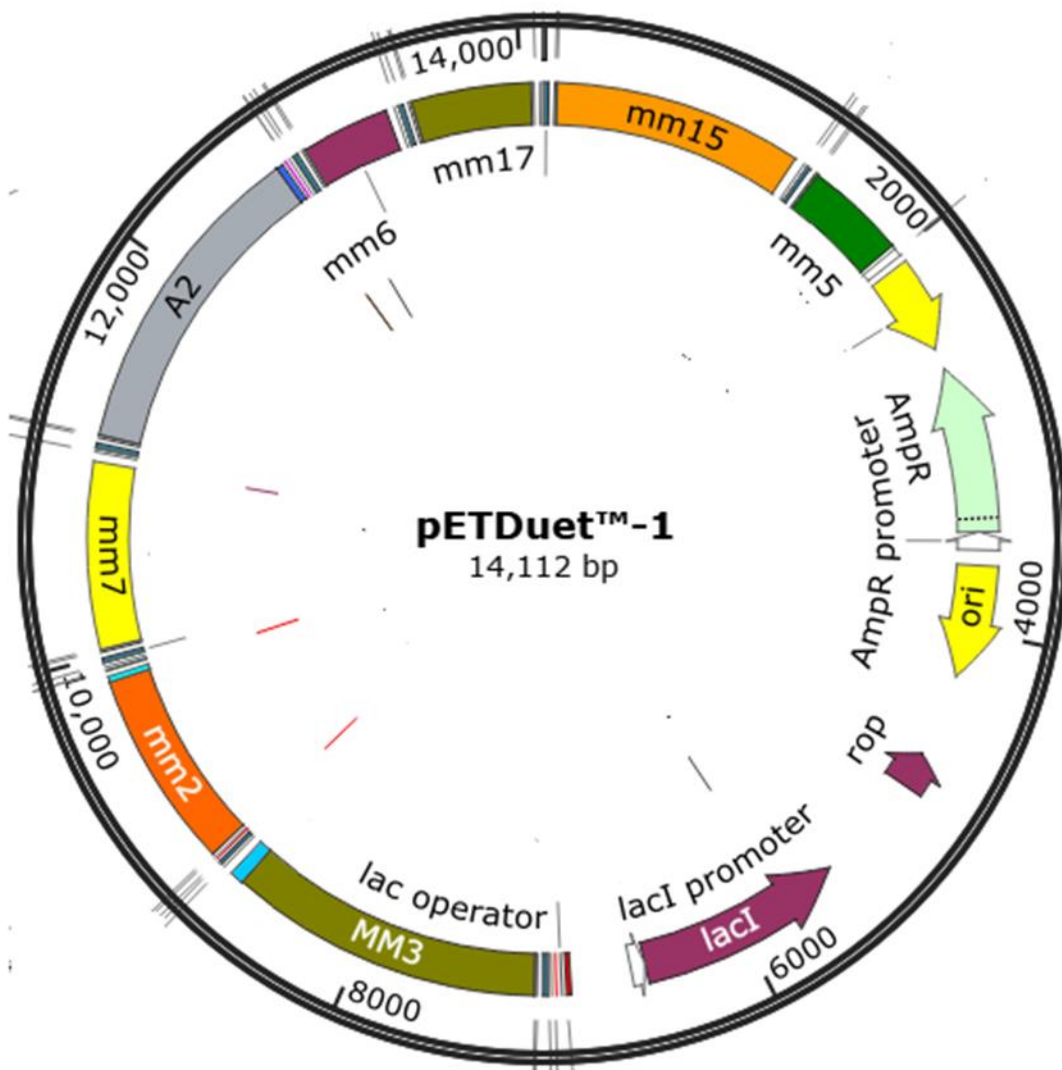

Figure 26) Map of *mmp* cluster

2.3.2) Cloning of mcr operon

The *mcr* operon contains *mcrA*, *mcrB*, *mcrG*, *mcrD* and *mcrC*. Recombinant McrA protein from *M. acetivorans* C2A previously was expressed in *E. coli* by lab mates in Dr. Mansoorabadi's lab and the expression was not successful due to lots of degradation of the expressed protein. It was decided to try an *mcrA* gene with optimized codons for expression in *E. coli*. The *mcr* operon was cloned in two steps, and they will be explained in detail in the next sections. The Gibson assembly method and SwaI restriction enzyme both were used to assemble *mcr* operon in one plasmid. The *mcrA* gene was cloned with a histidine tag. Two plasmids were assembled during a two steps process. Each of them was assigned a name and they can be found in Table 15.

Table 15) Two plasmids were assembled during the cloning of *mcr* operon. Each of them was assigned a name.

Plasmid	Name
pETDuet-*mmp3*-*mcrA*-optimized codon-C-His-tag	SH-8
pRSFDuet-SwaI-*mmp3*-*mcrA*-optimized codon-C-histag-*mcrGBDC*	*mcr* operon plasmid

2.3.2.A) Cloning design

First step: The pETDuet-*mmp3-mcrA*-C-His-tag plasmid was available in Dr. Mansoorabadi's lab. The *mcrA* gene was removed and the *mcrA* optimized codon for *E. coli* was inserted in the plasmid. The pETDuet-*mmp3-mcrA*-optimized codon-C-His-tag plasmid (SH-8) was assembled. (Figure 26)

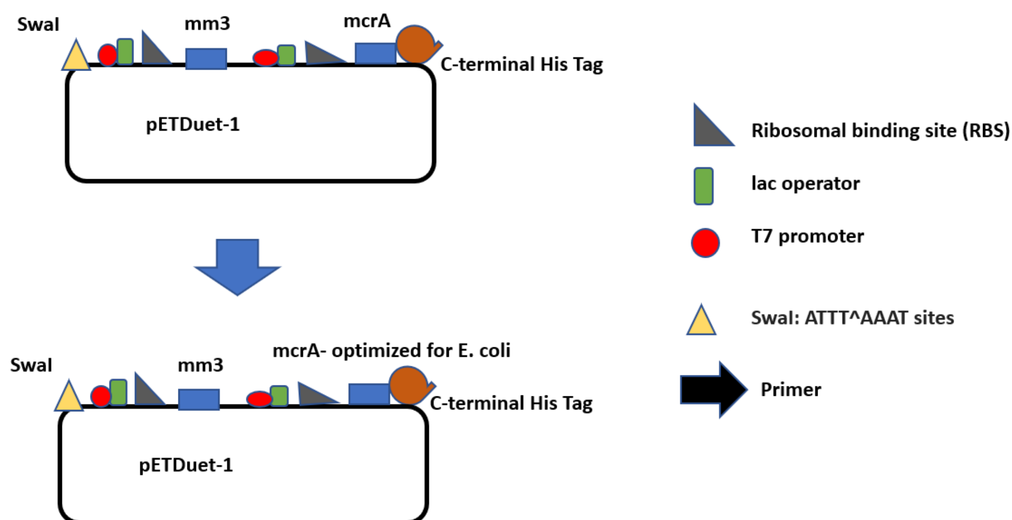

Figure 27) The first step of cloning *mcr* operon. Then, the mcrA gene was removed and the *mcrA* optimized codon for *E. coli* was inserted in the plasmid. pETDuet-*mmp3-mcrA*-optimized codon-C-His-tag plasmid was assembled.

Second step: pRSFDuet-SwaI-*mmp3-mcrA*-optimized codon-C-histag-*mcrGBDC* (*mcr* operon plasmid) was assembled from pRSFDuet-SwaI- *mcrGBDC*. (Figure 27)

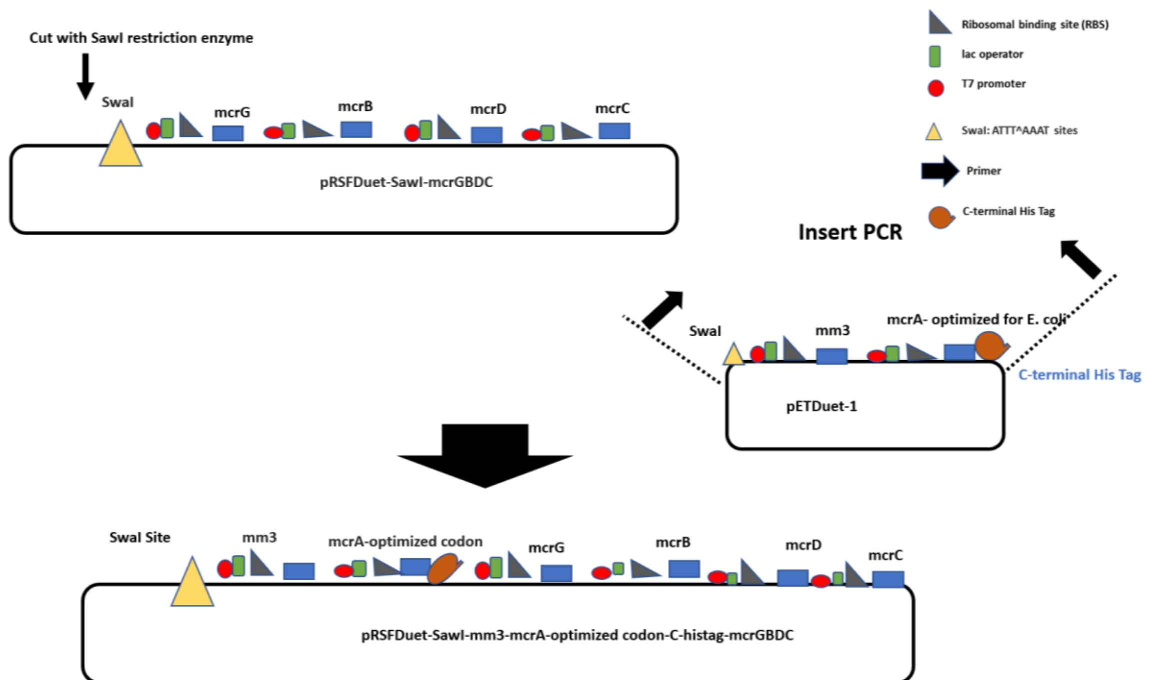

Figure 28) The second step of cloning the *mcr* operon. A PCR fragment containing the *mmp3* gene and *mcrA*-optimized codon for *E. coli*, their ribosome binding sites, lac operators and promoters, and SwaI restriction site was inserted into pRSFDuet-SwaI- *mcrGBDC*. pRSFDuet-SwaI- *mcrGBDC* plasmid was linearized with the SwaI enzyme. The insertion of two new genes was upstream of the promoter of the *mcrG* gene. *mcrA*-optimized codon cloned with C-terminal his tag. pRSFDuet-SwaI- *mcrGBDC* plasmid was available in Dr. Mansoorabadi's lab.

2.3.2.B) Cloning procedure

For each assembled plasmid one name was assigned and they can be found in Table 15. The procedure for cloning them is as below:

A. Cloning of plasmid SH-8

The *mcrA*-optimized codon was amplified with primers GA-NEWMCRA-F and GA-NEWMCRA-R to be inserted into the second cloning site of the plasmid. The linear backbone was amplified from the pETDuet-*mmp3*-*mcrA*- C-His-tag plasmid with primers RE-SE-PET-HISTAQ-F and RE-SEC-PACY-R. The Gibson assembly mixture was prepared. The ratio of the DNA insert (pmol) to backbone DNA (pmol) was around 3. The rest of the procedure was the same as the cloning of *mmp15* in the first cloning site of pETDuet-1. Sequences of the primers can be found in Table 16. The design of the plasmid assembly can be found in Figure 26.

Table 16) Primers for cloning of SH-8

Primers names	5´ to 3´
GA-NEWMCRA-F	GTTAAGTATAAGAAGGAGATATACATATGGCAGCAGATATCTT
GA-NEWMCRA-R	TCGAGCTAGTGATGGTGATGATGGTGTTTTGCAGGAATAACCAG
RE-SE-PET-HISTAQ-F	CACCATCATCACCATC
RE-SEC-PACY-R	ATGTATATCTCCTTCTTATACTTAACTAATA

B. Cloning of *mcr* operon plasmid

The insert included SwaI-*mmp3*-*mcrA*-optimized codon-C-histag-from pETDuet-*mmp3*-*mcrA*-optimized codon-C-His-tag plasmid was amplified with primers GA-MM3MCRA-F and GA-MM3MCRA-R primers. The pRSFDuet-SwaI- *mcrGBDC* was linearized with SwaI enzyme and purified on a 1.2% FlashGel DNA Cassette. The Gibson assembly mixture was prepared. The ratio of insert DNA (pmol) to backbone DNA (pmol) was around 5. For the verification of the successful insert of the fragment, the assembled plasmids and template plasmids both were linearized with the SwaI enzyme and then their size was compared. The samples were sent for sequencing. Sequences of primers can be found in Table 17. The design of the plasmid assembly can be found in Figure 27. The map is shown in Figure 29.

Table 17) Primers for cloning *mcr* operon plasmid

Primers names	5′ to 3′
GA-MM3MCRA-F	CGCTCTCCCTTATGCGACTCATGCGTCCGGCGTAG
GA-MM3MCRA-R	GTATTAATTTCCTAATGCAGTGCTAGTTATTGCTCAGCGG

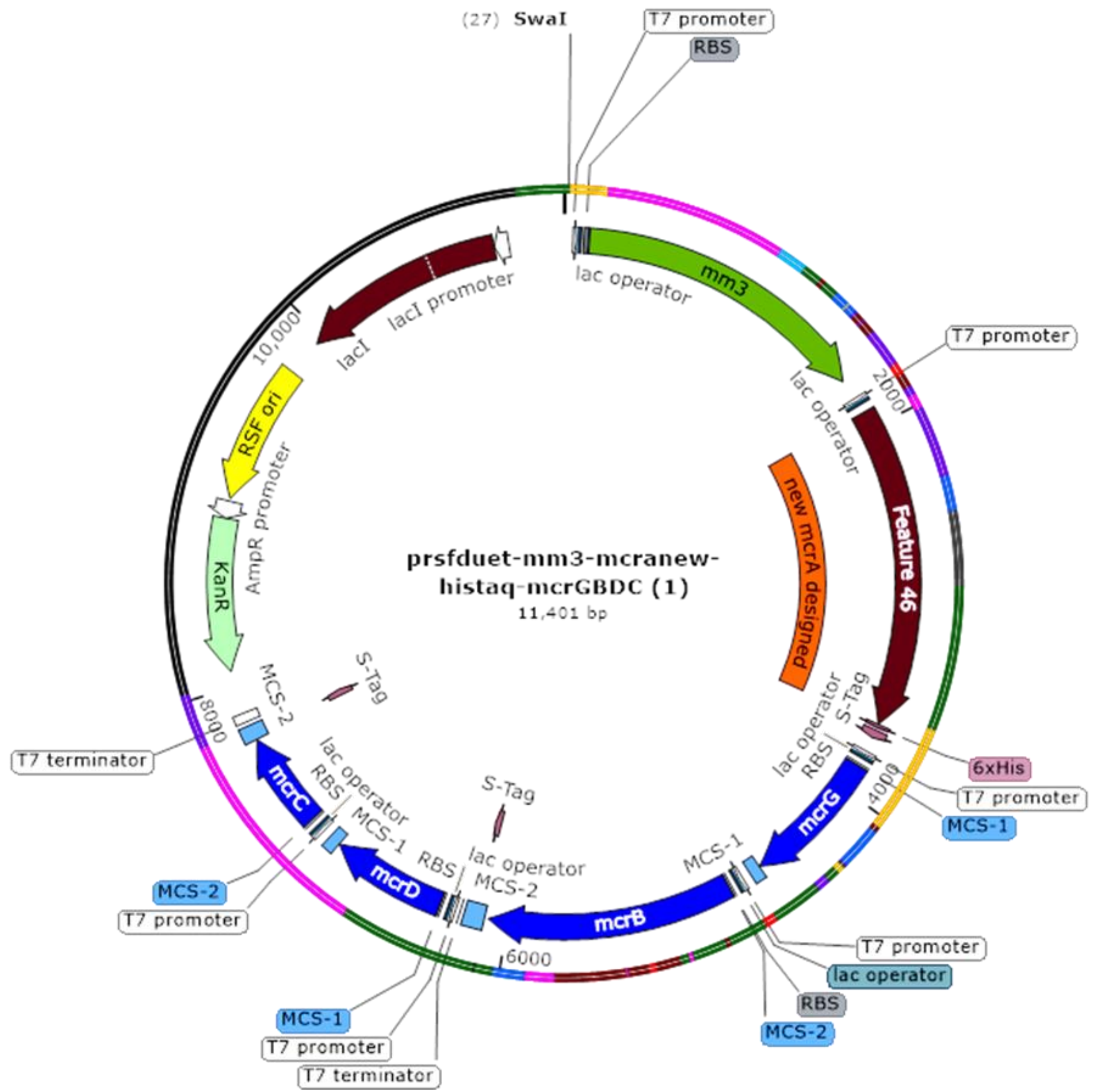

Figure 29) Map of *mcr* plasmid

2.3.3) Transformation into E. coli C41(DE3)

E. coli C41(DE3) cells were selected as hosts for the expression of the *mmp* cluster plasmid and the *mcr* operon plasmid. The *mmp* cluster contains 8 genes of interest and the *mcr* operon plasmids contain 6 genes of interest. The expression of 8 genes together or 6 genes together can be toxic for the cells. The C41(DE3) cells are designed to tolerate the expression of recombinant proteins. For more details check section 1.1.22.

Procedure:

A Microcentrifuge tube containing plasmids was placed on ice. C41(DE3) competent cells were kept in a -80 °C freezer. The tube of competent cells was transferred to the ice to thaw them for 10 min. 5 µl of plasmid was added to the competent cells, followed by mixing and incubation on ice for 30 minutes. The tube was transferred to a 42° C water bath for 45 seconds. It was transferred back to the ice for 10 min. 500 µl of Super Optimal broth (SOC) was added to the tube. It was shaken for 1 hour at 250 g at 37° C. 200 µl was transferred to an LB agar plate with the appropriate antibiotic and incubated overnight at 37°C.

2.3.4) Double transformation

The C41(DE3) cells harboring the *mcr* operon plasmid were grown in 5 mL LB media with added kanamycin (100mg/ mL). Competent cells were made from the cells harboring *mcr* operon with the common calcium chloride method.[84,85] In the next step, the *mmp* cluster plasmid was transformed into the competent cells (see section 2.3.3). The cells harboring *mcr* operon plasmid and *mmp* cluster plasmid were grown in 5 mL LB media containing kanamycin (100 mg/mL) and ampicillin (100 mg/mL).

2.3.5) Heterologous expression in E. coli C41(DE3) cells

2.3.5.1) Expression of *mmp* cluster

First step: *E. coli* C41(DE3) cells harboring *mmp* plasmid were grown in 4 L of broth containing 5 g NaCl, 10 g tryptone, and 5 g yeast extract per L with ampicillin (1 mL, 100 ng/mL) at 37 °C in a shaking incubator at 200 *g* until OD_{600} reached 0.5. Then, IPTG (0.5-1 mL, 1 M), $FeCl_3$ (0.5 mL, 1 M), and l-cysteine hydrochloride (0.2 g) were added. The temperature of the incubator was changed to room temperature. The cultures were grown for more than 20 hours at 25 °C at 100 g.

The second (anaerobic) step: Culture from the last step was transferred into an anaerobic growing container and placed on ice and purged with argon gas for a few hours. Then, it was transferred into the cold room and incubated over night before harvesting (Figure 28).

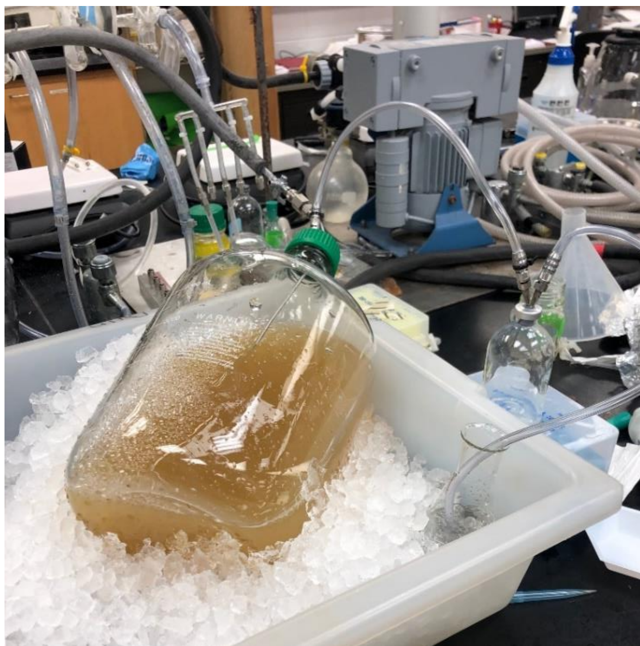

Figure 30) The second (anaerobic) step. The *E. coli* cells harboring plasmid were purged with argon gas on ice for a few hours in anaerobic conditions.

2.3.5.2) Expression of *mcr* cluster

Mcr cluster genes were expressed in *E. coli* C41(DE3) cells with the same procedure in 2.3.5.1 section except that kanamycin was used instead of ampicillin.

2.3.5.3) Co-expression of *mcr* operon genes and *mcr* cluster genes

Mmp cluster genes and *mcr* operon genes were co-expressed in *E. coli* C41(DE3) cells with the same procedure in the first step of 2.3.5.1 section except for that kanamycin and ampicillin both were used as antibiotics. The second step was not performed.

2.3.6) Purification with ATP agarose resin

Buffers: Buffer A or wash buffer contains 50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$ (pH 7.6). The elution buffer contains 50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM ATP (pH 7.6).: ATP disodium trihydrate (0.3 g) was added to 100 mL buffer A. This solution is not stable and must be prepared immediately before each purification.

Procedure:

Cells were harvested by centrifugation at 5,000 × *g* for 30 min at 4 °C. Pellets were resuspended in buffer A (100 mL per 4 L of cell culture). Protease inhibitor (100 times dilution, 1 µL protease /1000 µL cell lysis buffer) was added to the lysis buffer. The sample was sonicated on ice for 3 cycles of 3 min 70% output power, 5 sec on/5 sec off pulses. The cell debris was removed by centrifugation at 35,000 × *g* for 30 min at 4 °C. ATP agarose resin kept at 4 °C in a storage buffer (10 mM Tris/300 mM NaCl/1 mM EDTA, pH 8.0) was transferred into a disposable column and was washed a few times with 50 mL ice-cold buffer A. Then, the resin is added to the cell extract and incubated on ice for 2-3 hours while stirring gently. Then, the mixture is transferred into the disposable column, and the liquid is allowed to drip out. The column is washed a few times with ice-cold buffer A to remove unbound or loosely adsorbed material. The column outlet was capped, and 1 mL of ice-cold fresh elution buffer was added. After 30 min of incubation of the column on ice, the elution fraction was collected. This step was repeated 3 times. Four elution samples were collected.

2.3.7) Purification of his-tag proteins with Ni-Sepharose column

Buffer A: 50 mM Tris-HCl, 100 mM NaCl, pH 7.5

Buffer B: 50 mM Tris HCl, 100 mM NaCl, and 500 mM, pH 7.5

Procedure:

The cell pellet was resuspended in buffer A and lysed by sonication four times (60% Amplitude, 3.5 min, 0.5 on/off pulse). The lysates were centrifuged at 35,000 *g* for 30 min at 4 ºC. The supernatant was filtered using a 0.2 µM syringe filter and loaded on the 5 mL Ni-Sepharose column. A linear imidazole gradient from 0% to 100% buffer B was used to elute the protein.

2.3.8) Mass spectrometry

This step was performed by Dr. Chau-wen Chou from the mass spectrometry center of the University of Georgia according to the procedure below.

The purified proteins were run on 10% SDS-PAGE gels and then stained with AquaStain (Bulldog Bio). For In-gel trypsin digestion, the gel bands were cut into small pieces and then rinsed twice with 50% acetonitrile/20 mM ammonium bicarbonate (pH ~ 7.5-8). The gel pieces were dehydrated by adding 100% of acetonitrile and dried in a Speed Vac. Various amounts of a trypsin solution (0.01 µg/µL in 20 mM ammonium bicarbonate) were added until the gel pieces absorbed the solution. The samples were incubated at 37 °C overnight. The tryptic peptides were extracted from gel pieces by incubating twice with 50% acetonitrile/0.1% formic acid. The extracts were dried by a SpeedVac. A similar protocol was used for in-gel pepsin digestion. After the gel pieces were rinsed with 50% acetonitrile/20 mM ammonium bicarbonate to destain, the gel pieces were

rinsed with 0.1% formic acid twice before dehydration with 100% acetonitrile. Sufficient pepsin solution (Promega, 0.02 mg/mL in 0.04 M HCl) was added to cover the gel pieces. The samples were digested at 37 °C overnight (16-18 hours). The peptides were extracted with 50% acetonitrile in water. For in-solution trypsin digestion, samples were diluted with 20 mM ammonium bicarbonate to 0.5-1 g/L and supplemented with dithiothreitol at a final concentration of 10 mM. The samples were incubated at 100 °C for 5-10 minutes and allowed to cool to room temperature. The proteins were then digested with trypsin at the ratio of 50:1, protein to trypsin (w/w) overnight at 37 °C. The sample was then dried in a Vacufuge™.

For protein identification, the peptide mass fingerprinting (PMF) of gel bands was analyzed by a Bruker Autoflex Matrix-Assisted Laser Desorption Ionization (MALDI) Time-of-Flight (TOF) mass spectrometer. The matrix compound 2,5 dihydroxybenzoic acid (2,5-DHBA) was dissolved in 50% methanol to make a ~10 g/L solution. About 0.5-1 µl of the matrix solution and sample solutions (Tryptic peptides) were mixed and deposited on a metal plate and allowed to dry completely. For PTM analyses liquid chromatography was performed and the peaks analyzed with tandem mass spectrometry (LC-MS/MS) on a Thermo-Fisher LTQ Orbitrap Elite Mass Spectrometer coupled with a Proxeon Easy NanoLC system (Waltham, MA). The peptides were resuspended in 0.1% formic acid and then loaded into a reversed-phase column (self-packed column/emitter with 200 Å 5 µM Bruker MagicAQ C18 resin), then directly eluted into the mass spectrometer. Briefly, the two-buffer gradient elution (0.1% formic acid as buffer A and 99.9% acetonitrile with 0.1% formic acid as buffer B) started with 5% B, held at 5% B for 2 minutes, then increased to 25% B in 60 minutes, to 40% B in 10 minutes, and to 95% B in 10 minutes. The data-dependent acquisition (DDA) method was used to acquire MS data. A survey MS scan was acquired first, and then the top 5 ions in the MS scan were selected for the following CID and

HCD MS/MS analysis. Both MS and MS/MS scans were acquired by Orbitrap at the resolutions of 120,000 and 30,000, respectively. Data were acquired using Xcalibur software (version 2.2, Thermo Fisher Scientific). The protein identification and modification characterization were performed using Thermo Proteome Discoverer (version 1.3/1.4/2.2) with Mascot (Matrix Science) or SEQUEST (Thermo) programs. The spectra of modified peptides were inspected further to verify the accuracy of the assignments.

2.3.9) Western blotting

Tris-buffered saline (10 x TBS): 200 mM Tris, 5 M NaCl (pH 7.5).

Tris-buffered saline (1 x TBS): 20 mM Tris, 500 mM NaCl (pH 7.5).

TTBS (wash buffer): 20 mM Tris, 500 mM NaCl, 0.1 % Tween-20 (pH 7.5).

Blocking buffer (3 % gelatin-TBS): 20mM Tris, 500 mM NaCl, and 3 % gelatin (pH 7.5): Gelatin (3 g) was added to 10X TBS (10 mL) and then diluted to 100 mL with DI water. The bottle was transferred into the 50 °C water bath with gentle shaking until all gelatin dissolved then stored in the refrigerator. The buffer was reheated at 50 °C before use.

Antibody buffer (1% gelatin-TTBS): 20mM Tris, 500 mM NaCl, 1% gelatin, 0.1 % Tween-20 (pH 7.5): Gelatin (2 g) was added to TTBS (200 mL). The bottle was transferred into the 50 °C water bath with gentle shaking until all gelatin dissolved then stored in the refrigerator. Buffer was heated at 50 °C before use

Procedure:

All *E. coli* cells with a plasmid harboring histidine tag recombinant proteins were grown. Cells were harvested by centrifugation at 5,000 *g* for 20 min at 4° C and resuspended in 50 mM Tris-HCl, and 100 mM NaCl [pH 7.6]. Cells were lysed by sonication for 4 cycles of 5 sec ON/OFF for 3 min and the duty cycle set at 40%. The cell debris was removed from the lysate by centrifuging at 35,000 *g* for 20 min and the supernatant was collected. Proteins were separated on 12% SDS-PAGE gels and then transferred onto a soaked nitrocellulose membrane. Nonspecific binding was blocked with 3% gelatin-TBS blocking buffer for 1.5 h at room temperature. The membranes were then incubated with primary antibody against Histidine tag from (2:1,000 dilution) for 3 h at room temperature, washed three times for 5 min with TTBS. The membranes were incubated with a secondary antibody (Alkaline phosphatase detection, BIO-RAD) for 1 h at room temperature. The membranes were washed three times with TTBS. After additional washing with TBS, the membranes were developed by using an Alkaline phosphatase (AP) color development kit (BIO-RAD).

2.4) Results

2.4.1) Mmp cluster cloning, expression, purification

A pETDuet vector encoding the *mmp* cluster was constructed successfully as confirmed by DNA sequencing. The map can be found in Figure 29. The assembled plasmid was transformed into *E. coli* C41(DE3) cells. The A2 component protein has an affinity for ATP affinity and it was purified with N^6-(6-amino)hexyl-ATP-agarose (6AH-ATP-agarose) from Jena Bioscience.[86]

In the first round, the cells were not incubated under argon and the proteins were purified under air. Figure 30 shows the SDS-PAGE result. The gel shows two prominent bands at ~60 and ~50 kDa. Mass spectrometry finger printing showed them to be both due to A2. The size of A2 protein is 59.64 kDa and it has ATP affinity. As expected, a dominant band at ~60 was observed for it. Probably, the band at 50 kDa is for the degradation of A2. The complete purified sample was also analyzed by mass spectrometry. The result showed that component A2, Mmp7, Mmp17, Mmp6, Mmp5, Mmp15, and Mmp2 proteins are present in the sample and Mmp3 was absent. These extra proteins are available in the purified sample due to protein-protein interactions and they are co-purified with A2. The raw data is shown in Table A2.1 in appendix chapter 2. Table 18 contain information about the protein present and their molecular weights

In the second round, the *E. coli* C41 cells harboring the *mmp* cluster plasmid were purged with and incubated under argon gas before harvest and subsequently purified under the exclusion of air. Under these conditions, the sample has a yellow-brown color. The UV-vis spectrum before and after the addition of dithionite (DIT) to the sample is shown in Figure 31. The UV-vis spectrum is indicative of the presence of a $[4Fe\text{-}4S]^{2+}$ or $[3Fe\text{-}4S]^{+}$ cluster, with an absorption band at around 400 nm that loses intensity upon reduction by DIT. Figure 33 shows the EPR spectrum of the sample reduced with dithionite. The spectrum indicates the presence of a $[4Fe\text{-}4S]^{+}$ cluster with the typical g = 1.94 value for the g_y peak. The signal intensity of the EPR signal is low. The absorption spectrum shows that not all clusters get reduced. It is possible that the midpoint potential of the clusters is close too or lower than that of dithionite. The sample was run on 12% SDS-PAGE gel (Figure 32). The SDS-PAGE looks like the one in Figure 30 with the two intense A2 bands. Mass spectrometry analysis of the whole sample showed that mmp3 is now present. The raw data can be found in Table A2.2 in appendix chapter 2

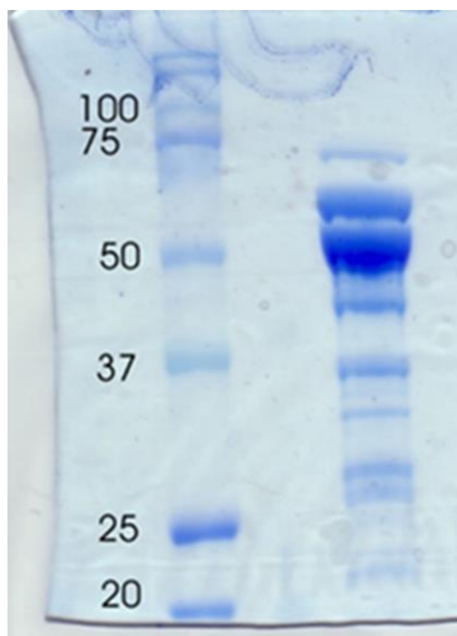

Figure 31) SDS-PAGE (12%) result of the Mmp cluster sample after purification with ATP-agarose.

Table 18) Protein present in the aerobic Mmp purified sample. The complete purified sample was also analyzed by mass spectrometry. The raw data is shown in Figure A2.1 in appendix chapter 2.

No	Name of the gene	NCBI Reference Sequence	k Da
1	Methyl coenzyme M reductase system, component A2	WP_011023893.1	59.64
2	Methanogenesis marker 7 (Mmp7)	WP_011023887.1	34.36
4	Methanogenesis marker 6 (Mmp6)	WP_048065869.1	17.83
5	Methanogenesis marker 5 (Mmp5)	WP_048066559.1	18.67
6	Methanogenesis marker 15 (Mmp15)	WP_011023889.1	44.02
7	Methanogenesis marker 17 (Mmp17)	WP_011023888.1	22.75
8	Methanogenesis marker 2 (Mmp2)	WP_011024078.1	35.59

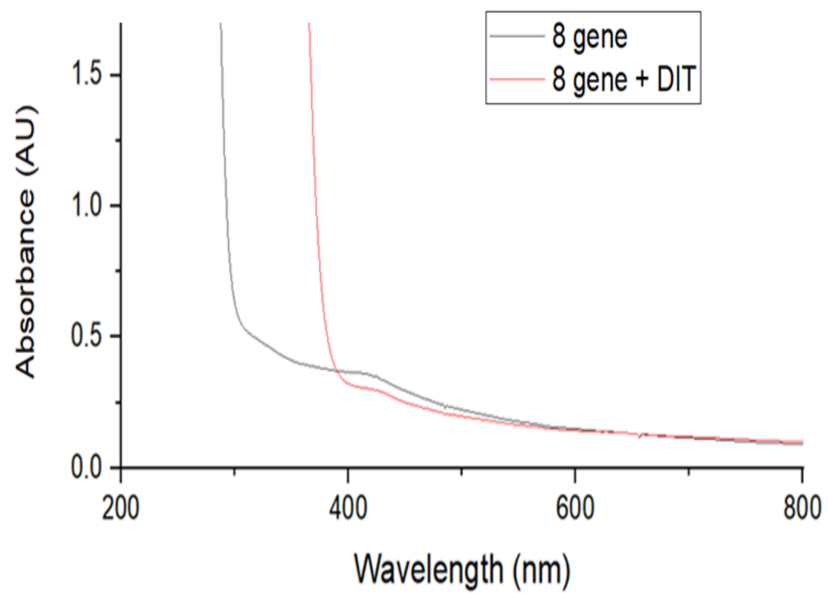

Figure 32) The UV-visible spectrum of the anaerobically Mmp cluster purified protein complex in the absence (black curve) and presence (red curve) of dithionite.

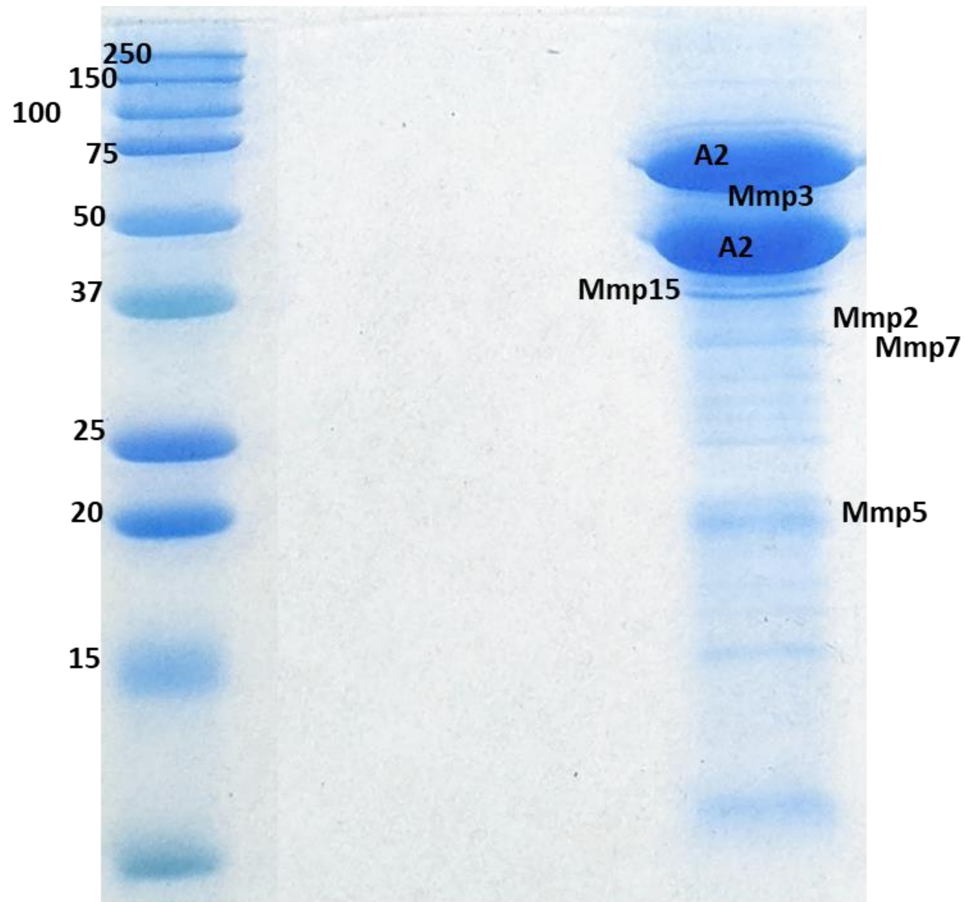

Figure 33) The SDS-PAGE gel of the Mmp cluster purification anaerobically. Cells were purged with argon gas before harvesting.

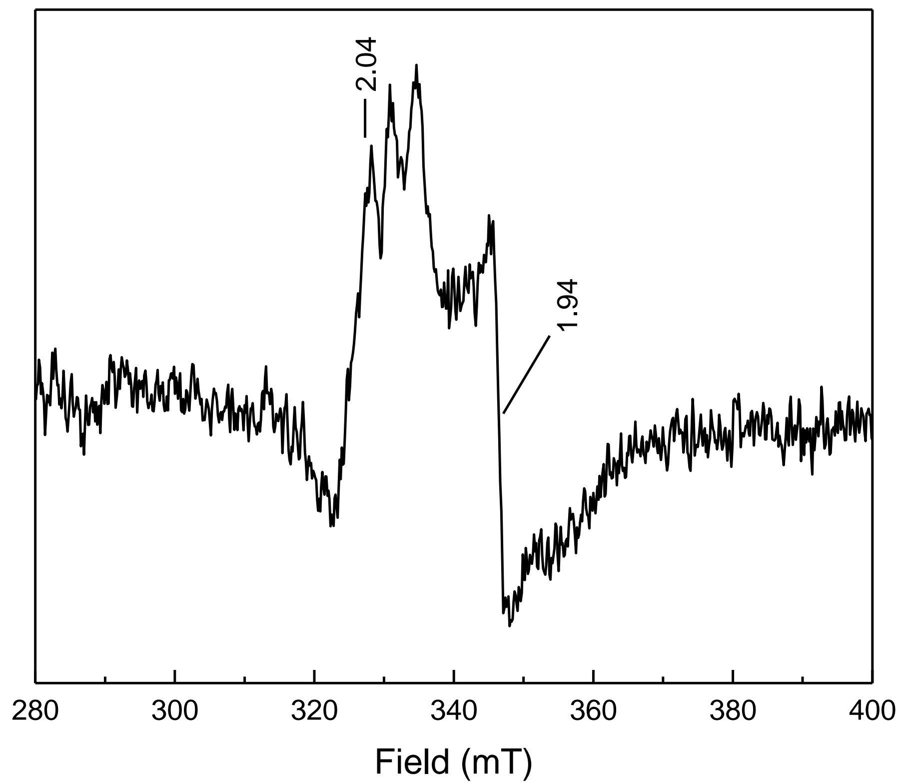

Figure 34) EPR of the Mmp clusters sample reduced with 5 mM dithionite. Measuring conditions: Power, 20 dB; Temperature 8.6 K; Frequency 9384 MHz.

2.4.2) Mcr Operon

The *mcr* operon-*mmp3* plasmid was cloned successfully from pETDUET-*mcrBGDC* and approved by DNA sequencing. The assembled plasmid was transformed into *E. coli* C41(DE3) cells. 4 L cell culture was grown. The heterologously produced proteins were purified with a Ni-Sepharose column under the exclusion of air. The FPLC chromatogram for McrA-C-histidine tag purification with Ni-Sepharose column is shown in Figure 35. Figure 36 shows the SDS-PAGE result. The more intense bands (1-4) were cut out and analyzed by MALDI. All four were from *E. coli*. Mass spectrometry analysis of the complete sample showed the presence of McrC and McrD. The rest of the protein present in the sample was from *E. coli*.

Since the McrA protein was cloned with a C-terminus histidine tag, the purified sample and pellet were tested with the western blot experiment, but no trace of the McrA-C-histidine tag was detected. McrA was not expressed. McrA might be toxic for the host. Mcr operon plasmid has repetitive DNA sequences. These could result in the deletion of the gene and clone instability.[87]

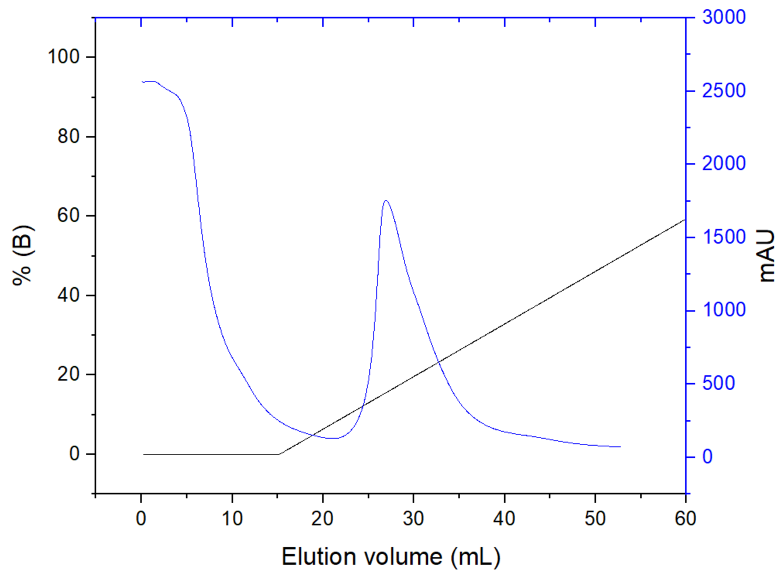

Figure 35) FPLC chromatogram for McrA-C-histidine tag purification with Ni-Sepharose column. A linear imidazole gradient from 0% to 100% buffer B was used to elute the protein.

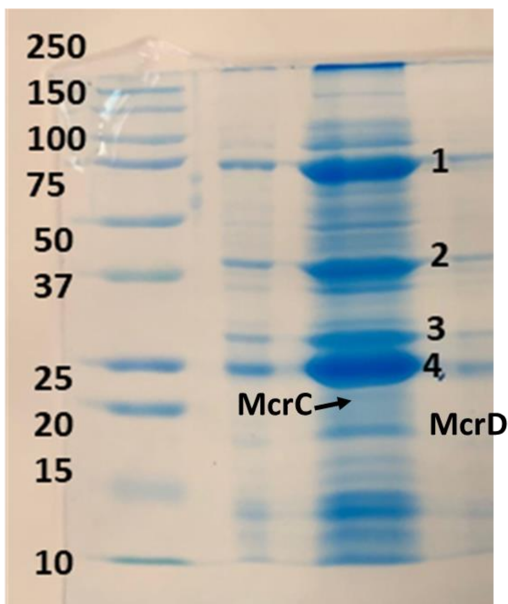

Figure 36) McrA with a C-terminal histidine tag was purified anaerobically and run in 12% SDS-PAGE gel. The more intense bands (1-4) were cut out and analyzed by MALDI. All four were from *E. coli*.

2.4.3) *Co-expression of the mcr operon and mmp cluster proteins*

Both *mmp* cluster plasmid and *mcr* operon plasmid were transformed into *E. coli* C41 cells with the double transformation method mentioned as described in 2.3.4 section. pETDuet-1 and pRSFDuet-1 plasmids are compatible, and they can co-exist in the same host cell. (See 1.1.21 section for more details) 4-liter cells harboring both plasmids were grown and purified with a Ni-Sepharose column. The FPLC chromatogram is shown in Figure 37. Mass spectrometry analysis of the complete sample showed that all the present proteins in the sample were from *E. coli*. A western blot experiment was performed with the purified sample, but no bands for McrA were detected.

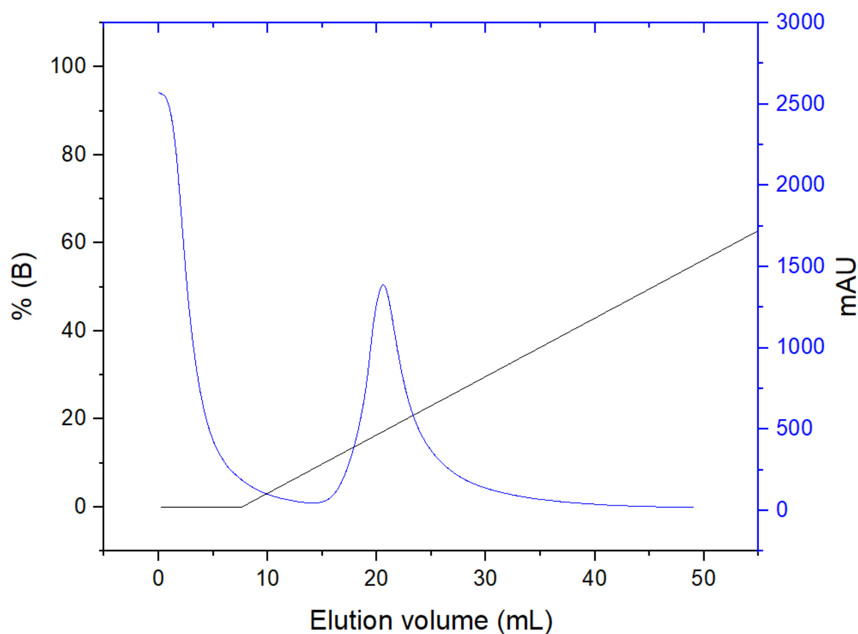

Figure 37) FPLC chromatogram for purification of the 6xHis-tagged protein on a Ni-Sepharose column. The McrA contained the C-terminal His-tag. In this experiment the *mcr* operon and *mmp* cluster genes were co-expressed. A linear imidazole gradient from 0% to 100% buffer B was used to elute the protein.

2.5) Discussion

Methanogenesis marker proteins are unique to methanogenic archaea. There is a conserved cluster of methanogenesis marker genes in some methanogens. It was not clear if they form a protein complex since they are not always grouped. It is also not clear what their function would be. In the prokaryotic genome, genes are not usually distributed randomly, they are organized. A cluster of genes could have functions that is somehow related and form a protein complex or associate in the same biological pathway (see 2.1 section for more details). Some of the Mmp cluster proteins are present in the isolated A3a component. It can be hypothesized that Mmp cluster proteins could be important for the activity of Mcr or the assembly of Mcr.

The first goal of this research was to check if the *mmp* cluster codes for a large complex. *Mmp5*, *mmp6*, *mmp17*, *mmp7*, *mmp3, mmp2*, *mmp15,* and *atwA* genes from the *mmp* cluster of *M. acetivorans* were cloned in the pETDuet-1 plasmid. Each gene was cloned with a separate ribosome binding site and T7 promoter. The total length of the 8 genes with promoters and ribosome binding sites is around 7,000 bp. The total length of the assembled plasmid is around 14,000 bp. Seven plasmids were assembled during four steps process to use for cloning of 8 genes plasmids. The plasmid of interest was pETDuet-*mmp7-atwA-mmp3-mmp2-mmp6-mmp17-mmp5-mmp15* and named *mmp* cluster plasmid. To increase the efficiency of assembly with the Gibson assembly method, 0.2 µL DMSO was added to the Gibson assembly mixture. DMSO help for the assembly of the fragments with a high GC content of the overlap area (≤ 40%). The ratio of insert to the backbone is important too. Using the SwaI restriction site helped to make large linear fragments from large plasmids without the need to do PCR and avoid possible mutations.

E. coli C41(DE3) cells as a host for expression worked well for co-expression of 8 genes. 4 -liters of cells harboring *mmp* plasmid were grown. A2 component has ATP hydrolase activity

and ATP resin was chosen for the purification. This would avoid having a tag on a position that could interfere with the protein cluster assembly. All 8 proteins are present, A2, Mmp 2, 3, 5, 6, 7, 15, and 17 when the cells are incubated under argon before harvest, and the purification is done under the exclusion of oxygen. Mmp3 is not present when these precautions are not taken. Based on this study, the expression in *E. coli* was successful and a full complex was formed. When the cluster was purified anaerobically, Mmp3 was present in the sample. The anaerobically purified complex has a color and EPR shows the presence of $[4Fe\text{-}4S]^{2+}$. The aerobically purified complex has no color. This could indicate highly sensitive $[4Fe\text{-}4S]^{2+}$ that is present on the surface of the complex. It is not clear if the absence of clusters is directly responsible for the disappearance of Mmp3 or whether that is a side effect of other subunits not being folded properly when the lack a cluster.

Chapter 3 : Identification of A3a and characterization of component A2

Any attempts to use Mcr for the biological production of methane or oppositely to capture methane to make liquid biofuels will require a fundamental understanding of how the protein is expressed, modified, folded, and activated.[14] The study of Mcr is very slow. Upon opening the cells, the Mcr enzyme losses its activity even in a strictly anaerobic environment due to the very negative electron redox potential of Ni(I)F_{430}. When Mcr loses its activity, there is no method available to turn it to the active form again *in vitro*. This problem limits the study of Mcr. Every time researchers try to study the enzyme, freshly purified Mcr is needed. The native organism needs to be grown and then anaerobically harvested and purified. It is a very time-consuming and sensitive process. Even a little bit of oxygen leaking during harvest or purification can cause a loss of activity. Duin and coworkers were able to activate the different forms for Mcr $_{ox1}$, and Mcr $_{silent}$, with recombinant A2, isolated A3a, Mcr, MgATP, M-CoM, and dithiothreitol. 100% of Mcr_{ox1} and 65% of Mcr_{silent} were activated, as described in sections 1.1.14 to 1.1.16.

For the activation of Mcr, the reduction of Ni is necessary. For the reduction, it seems two requirements need to be fulfilled. First, an electron needs to be transferred from an electron source to the Ni of the F_{430}-bound enzyme. Maybe A3a works as an electron transfer system. Second, the electron needs to have enough potential to be able to reduce the Ni of the F_{430}-bound enzyme. An electron from electron sources such as H_2 or dithiothreitol has a higher midpoint potential and as a result, is not able to reduce Ni of F_{430} bound enzyme. Since ATP is required for the activation of Mcr, ATP-hydrolysis couple electron transfer could result in the formation of a low potential electron. In addition, electron bifurcation can form low-potential electrons. Utilization of both ATP and electron bifurcation may combine to form low-potential electrons capable of reducing the Ni in the enzyme.

A3a was isolated from methanogen cells with a four-column purification procedure. The four-column purification procedure was repeated multiple times, each time giving different results in composition and activating properties. Components might be lost in these long purification procedures. The question is what is the exact protein composition of A3a? What are the prosthetic groups present and what is their role in the activation process? By solving that, we might be able to understand how the whole protein complex and the activation process work.

A different approach is needed to determine the protein composition of A3a. Pull-down experiments with individual subunits could reveal protein partners and combined provide the full subunit composition of A3a. In this work, four genes we think are part of the A3a complex were chosen, including the genes for the iron-sulfur cluster protein (*FFP*), methanogenesis marker 7 (*mmp7*), ATP binding protein, and methyl-coenzyme M reductase I operon protein C (*mcrC*). Also included is *atwA* (gene for component A2) since it is essential for ATP-dependent activation. They were homologously expressed in *M. maripaludis* and then purified. Based on this research, several of the other protein partners of these proteins in the A3a complex were identified. The proteins were also tested for the presence of cofactors.

In the first attempt, all five genes were cloned one by one in pMEV4mTs with a histidine tag and histone promoter (P_{hmvA}). The assembled plasmids were transformed into *M. maripaludis.* The cells harboring plasmid with the *atwA* gene and *mmp7* gene did not grow at all (Table 19). The cells harboring plasmid with *mcrC*, *FFP*, and ATP binding proteins survived. Western blot experiment showed that the expression level of protein was very low or negligible. The ratio of impurities eluted during purification to the purified proteins was large. It was concluded that the histone promoter did not work. Due to the low expression levels, impurities become a large problem.

Table 19) Cloned genes with histone promoter and the histidine tag

No	Plasmid design	Did the *M. maripaludis cells* grow after transformation?
1	pMEV4mTs-N-His-tag-*mcrC*	Yes
2	pMEV4mTs-N-His-tag-ATP binding protein	Yes
3	pMEV4mTs-N-His-tag-*FFP*	Yes
4	pMEV4mTs-C-His-tag-*mmp7*	No
5	pMEV4mTs-C-His-tag-*atwA*	No

To alleviate this problem, the expression system was changed to a phosphate-regulated promoter and the tag was changed to a Flag-Strep$_2$ tag. In addition, Mmp7 and A2 proteins were co-expressed together since the pull-down experiment by a previous student indicated a close interaction between these two proteins in *M. marburgensis*. Transformation of all the assembled plasmids was successful and all cells harboring the cloned plasmids survived.

3.1) Materials

pMEV4mTs with histone promoter and histidine tag was a gift from the Whitman lab (UGA). pMEV4mTs with a phosphate regulated promoter and Flag-Strep2 tag was a gift from the Whitman lab (UGA). N6-(6-Amino)hexyl-ATP-Agarose (6AH-ATPAgarose) resin was purchased from Jena Bioscience. Phusion High-Fidelity DNA Polymerase (92,000 units/ml with 100 units) was purchased from New England Biolabs. NEBuilder HiFi DNA Assembly Cloning Kit was purchased from New England Biolabs. 1.2% FlashGel DNA Cassette and FlashGel DNA marker (100 bp – 4 kb) were purchased from Lonza. Z.N.A Gel Extraction Kits was purchased from Omega. EconoTaq PLUS GREEN 2X Master Mix was purchased from Thermo Scientific. Nanosep Centrifugal Devices Membrane 10 and 3K were purchased from Omega. Protease

Inhibitor Cocktail Tablets (cOmplete, Mini, EDTA-free) were purchased from Roche. Single antibody (histidine tag, Alkaline Phosphatase detection) was purchased from Sigma-Aldrich. Primary antibody (Histidine tag,) and Secondary Antibody (Alkaline phosphatase) were purchased from BIO-RAD. Alkaline phosphatase (AP) color development was purchased from BIO-RAD. Primary Antibody (6x-His Tag) and Secondary Antibody with Horseradish Peroxidase detection (HRP) and HRP color development (Metal Enhanced DAB Substrate Kit) were purchased from Invitrogen.

3.2) Experiments

3.2.1) Cloning

pMEV4mTs was selected for homologous expression of *mmp7*, *atwA*, *mcrC*, ATP binding protein, and *FFP* genes. More details about mentioned genes can be found in Table 20. The host was *M. maripaludis* S2. This plasmid can be recognized by both hosts: *E. coli* (ampicillin resistance gene) and *M. maripaludis cells* (puromycin resistance gene). However, genes of interest can only be expressed in *M. maripaludis.* The promoter was phosphate-regulated. Protein expression under the control of this promoter is regulated by the inorganic phosphate concentration of the media as described in section 1.1.19. *E. coli* was used as a host for the cloning and assembly of plasmids. For plasmid SH-13 and SH-14, two genes were cloned in the same plasmid, and they shared the same promoter. Assembled plasmids and their given names are found in Table 21.

Table 20) Information about five genes

No	Name of the gene	Length of gene	Old locus tag	NCBI Reference Sequence	Protein size (Kilodaltons)
1	Methyl coenzyme M reductase system, component *A2* (*atwA*)	1596	MMP0620	WP_011170564.1	59.5
2	Methanogenesis marker 7 (*mmp7*)	915	MMP0421	WP_011170365.1	34.97
3	Iron-sulfur cluster protein (*FFP*)	576	MMP0614	WP_011170558.1	21.02
4	methyl-coenzyme M reductase I operon protein C (*mcrC*)	597	MMP1557	WP_011171501.1	21.36
5	ATP binding protein	786	MMP0593	WP_011170537.1	27.42

Table 21) Five plasmids were assembled for *M. maripaludis* as the host.

Assembled plasmid	Given name
pMEV4mTs-N- Flag-Strep2 tag – *mcrC*- phosphate regulated promoter	SH-10
pMEV4mTs-N- Flag-$Strep_2$ tag- ATP binding protein- phosphate regulated promoter	SH-11
pMEV4mTs-N- Flag-$Strep_2$ tag- *FFP*- phosphate regulated promoter	SH-12
pMEV4mTs-C- Flag-Strep2 tag – *atwA* (CS1)- *mmp7* (CS2) -phosphate regulated promoter	SH-13
pMEV4mTs-C- Flag-Strep2 tag – *mmp7* (CS1)- atwA (CS2) -phosphate regulated promoter	SH-14

Cloning procedure:

For each assembled plasmid one name was assigned and they can be found in Table 21. The procedures for cloning them are as follows:

A. Cloning of plasmid SH-10

The *mcrC* gene was amplified from the chromosomal DNA of *M. maripaludis S*2 with primers STREP-MCRC-F and STREP-MCRC-R for insertion into the cloning site of pMEV4mTs-N-terminal strep Flag-$Strep_2$ tag plasmid with a phosphate-regulated promoter. The linear backbone

was amplified from pMEV4mTs-N-terminal strep Flag-$Strep_2$ tag plasmid with primers RE-STREP-NTAG-F and RE-STREP-NTAG-R. Phusion® High-Fidelity DNA Polymerase and GC buffer were used for the PCR. PCR samples were mixed with FlashGel™ loading dye and loaded into the tiers of the 1.2% FlashGel® DNA Cassette wells along with FlashGel™ DNA marker (100 bp – 4 kb) and the band with the correct size was isolated and DNA was extracted from the gel with E.Z.N.A.® Gel Extraction Kits and was eluted in the nuclease-free water and stored at -20 °C. The Gibson assembly mixture was prepared with DNA insert fragment, DNA backbone fragment, nuclease-free water, DMSO (0.2 µL), and Gibson Assembly Master Mix (2×) (10 µL). The ratio of insert DNA (pmol) to backbone DNA (pmol) was around 3. The Gibson assembly mixture was heated for 30 min at 50 °C. The mixture was put on ice and then transformed into the NEB 5-alpha competent *E. coli*. Two colonies were selected and grown in a 5 mL autoclaved LB medium with 8 µl ampicillin (100 ng/mL). Plasmids of each culture were isolated. PCR experiments were carried out to verify the successful insertion of the *mcrC* gene in the assembled plasmid. The verified plasmid was sent for DNA sequencing. Sequencing primers were pMEV4-F and pMEV4-R. Sequences of primers can be found in Table 22. The map is shown in figure 38.

Table 22) Primers for plasmid SH-10

Primers names	5′ to 3′
STREP-MCRC-F	GTCAGGTATGCCAGTAGGTAGAAAAGAGCAG
STREP-MCRC-R	CGCTACTAGTTTAACCTAAATGTTTTTTATGCTTTC
RE-STREP-NTAG-F	TTTAGGTTAAACTAGTAGCGGCCGCTGC
RE-STREP-NTAG-R	TACCTACTGGCATACCTGACTTTTCGAACTGGG
PMEV4-F	TCCTTCCTTCTTTCCTGCATACTT
PMEV4-R	CTGTCATGGAAGGTCGTCTCC

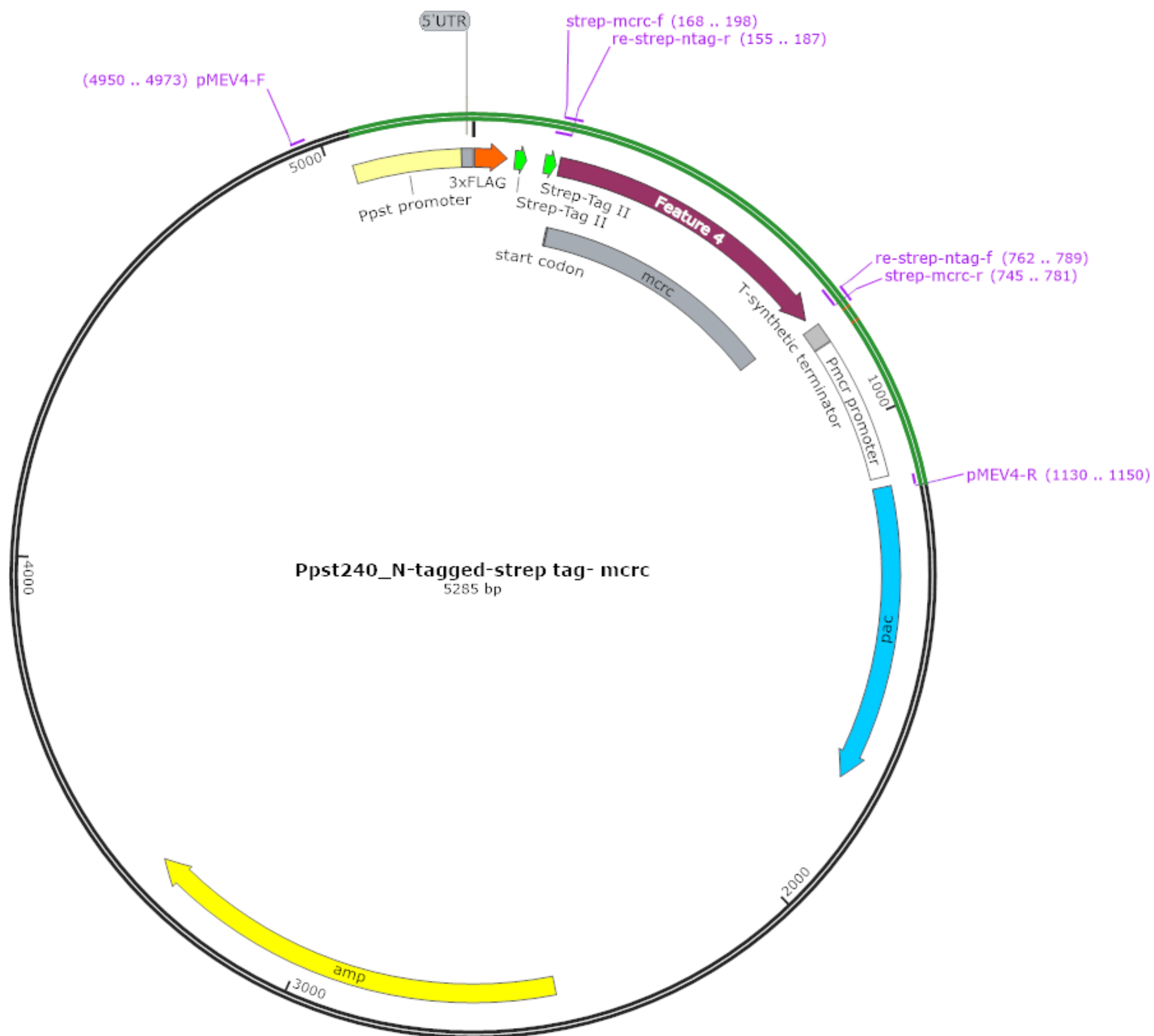

Figure 38) Map for plasmid SH-10

B. Cloning of plasmid SH-11

The ATP binding protein gene was amplified from the chromosomal DNA of *M. maripaludis* S2 with primers SDP_FWD and SDP_REV and for the cloning site of pMEV4mTs-N-terminal strep tag with phosphate promoter. The linear backbone was amplified from pMEV4mTs-N-terminal Flag-Strep2 tag plasmid with the phosphate-regulated promoter with primers backbone for SDP-FWD and backbone for SDP-REV. The rest of the procedure is the same as the cloning of the plasmid SH-10 and the ratio of the insert DNA fragment to the backbone DNA fragment was 3 pmol/1 pmol. Sequencing primers were pMEV4-F and pMEV4-R. Sequences of primers can be found in Table 23. The map is shown in Figure 39.

Table 23) Primers for Cloning plasmid SH-11

Primers names	5´ to 3´
SDP-FWD	AAAGTCAGGTATGGCTATAACAATTGCAG
SDP-REV	CGCTACTAGTTCATTTTTTCTTACCCCC
BACKBONE FOR SDP-FWD	GAAAAAATGAACTAGTAGCGGCCGCTGC
BACKBONE FOR SDP-REV	TTATAGCCATACCTGACTTTTCGAACTGGGG
PMEV4-F	TCCTTCCTTCTTTCCTGCATACTT
PMEV4-R	CTGTCATGGAAGGTCGTCTCC

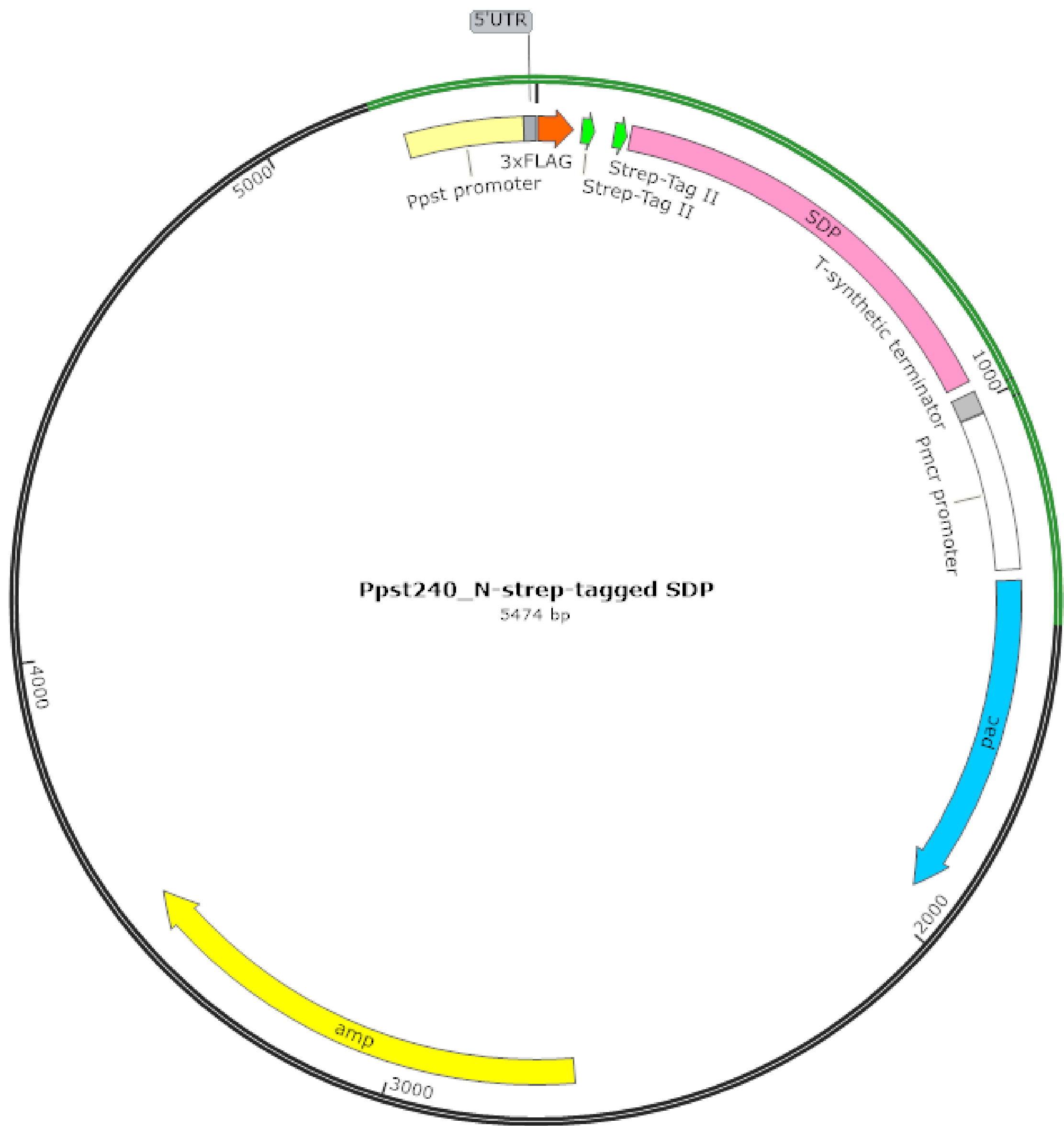

Figure 39) Map for plasmid SH-11

C. Cloning of Plasmid SH-12

The iron-sulfur cluster protein (*FFP*) gene was amplified from the chromosomal DNA of *M. maripaludis* S2 with FFP-STERPTAQ-REV and FFP-STERPTAQ-FWD primers and for the cloning site of pMEV4mTs-N-terminal strep tag with phosphate promoter. The linear backbone was amplified from pMEV4mTs-N-terminal Flag-$Strep_2$ tag plasmid with the phosphate-regulated promoter with BACKBONE-FFP-REV and BACKBONE-FFP-FWD primers backbone. The ratio of the insert DNA fragment to the backbone DNA fragment was 3 pmol /1 pmol. The rest of the procedure is the same as the cloning of plasmid SH-12. Sequencing primers were pMEV4-F and pMEV4-R. Sequences of primers can be found in Table 24. The map is shown in Figure 40.

Table 24) Primers for Cloning of plasmid SH-12

Primers names	5′ to 3′
BACKBONE-FFP-REV	CTACTTTCAAACCTGACTTTTCGAACTGGGG
BACKBONE-FFP-FWD	TAACGAATAAACTAGTAGCGGCCGCTGC
FFP-STERPTAQ-FWD	AAAGTCAGGTTTGAAAGTAGTTGCATTTAAC
FFP-STERPTAQ-REV	CGCTACTAGTTTATTCGTTAATTTTTTTCAAAATCC
PMEV4-F	TCCTTCCTTCTTTCCTGCATACTT
PMEV4-R	CTGTCATGGAAGGTCGTCTCC

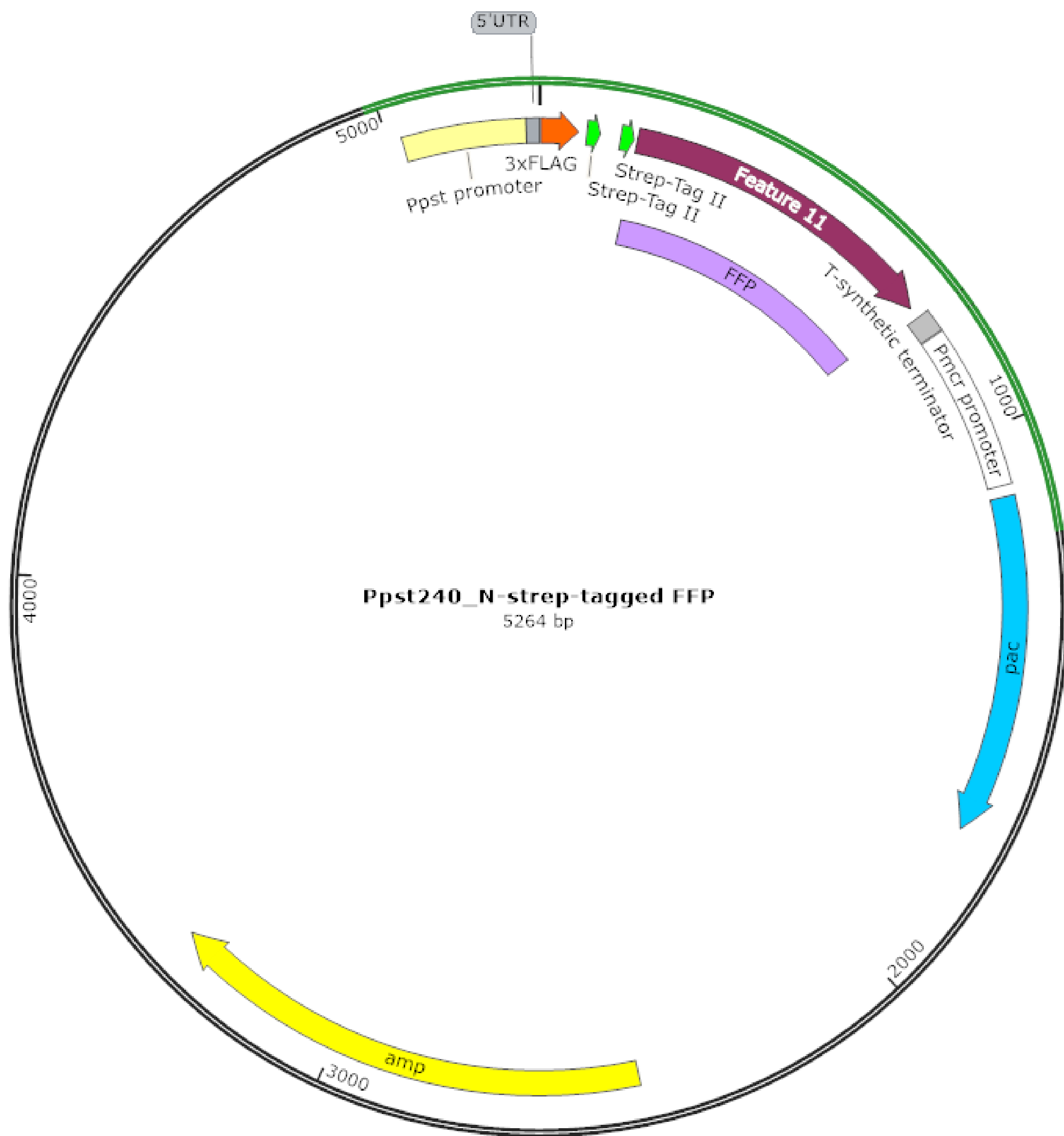

Figure 40) Map of plasmid SH-12

D. Cloning Plasmid SH-13

This part involved four-fragment cloning. The atwA gene was amplified from the chromosomal DNA of *M. maripaludis* S2 with primers 1-A2-FWD and 1-A2-REV. The *mmp7* gene was amplified with 1-MM7-FWD and 1-MM7-REV primers from the chromosomal DNA of *M. maripaludis* S2. The C-terminal Flag-$Strep_2$ tag was amplified with 1-TAG-FWD and 1-TAG-REV from pMEV4mTs-C-terminal Flag-$Strep_2$ tag. The vector was amplified from pMEV4mTs-C-terminal Flag-$Strep_2$ tag plasmid with the phosphate-regulated promoter with 1-NEWFRAGMENT-REV a 1-NEWFRAGMENT-FWD. All four fragments were mixed for the Gibson assembly method. The ratio of the four fragments was 1:1:1:1. Sequencing primers were pMEV4-F and pMEV4-R. Sequences of primers can be found in Table 25. The map is shown in Figure 41.

Table 25) Primers for cloning of plasmid SH-13

Primers names	5′ to 3′
1-A2-FWD	GGTGTCTCATATGTTACTTTTGGAAGTGAAAAATG
1-A2-REV	CCTTGTAGTCATGGCCGATCATTTCATC
1-TAG-FWD	GATCGGCCATGACTACAAGGACCACGAC
1-TAG-REV	CCTTAAAGGTTTACTTTTCGAACTGGGG
1-NEWFRAGMENT-REV	AAAGTAACATATGAGACACCTCCCAGGTTTATG
1-NEWFRAGMENT-FWD	TAAAGCTTAAACTAGTAGCGGCCGCTGC
1-MM7-FWD	CGAAAAGTAAACCTTTAAGGTGGAAAAG
1-MM7-REV	CGCTACTAGTTTAAGCTTTATTTTCATCCGTTTTAAG

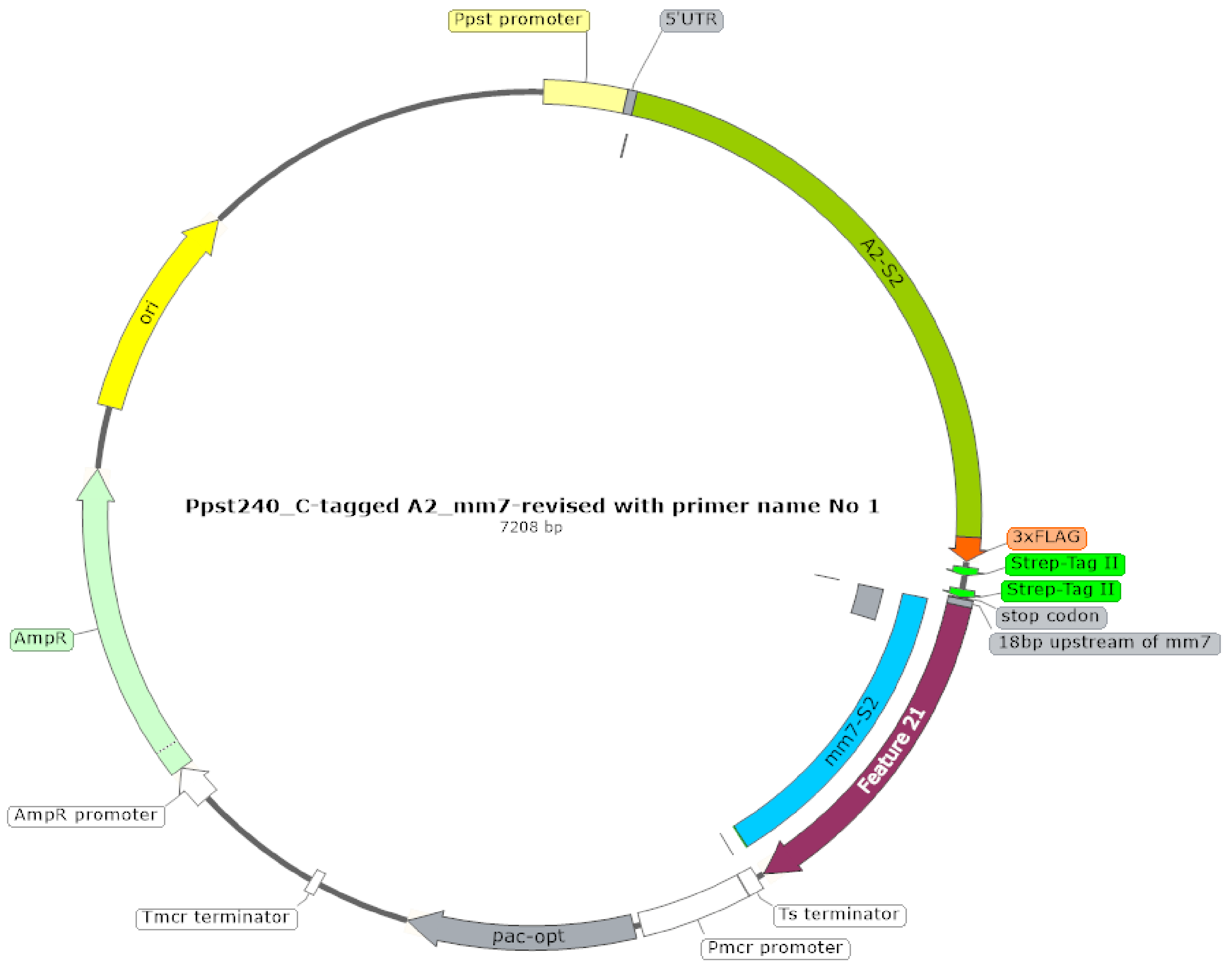

Figure 41) Map of plasmid SH-13

E. Cloning plasmid SH-14

This plasmid was assembled in two steps. In the first step, the *mmp7* gene was cloned in the only cloning site of the pMEV4mTs. And then, the *atwA* gene was cloned downstream of *mmp7* in the plasmid. The plasmid is shown in Figure 42.

First step:

The *mmp7* gene was cloned in the cloning site of the pMEV4mTs-C-terminal Flag-Strep$_2$ tag. The *mmp7* gene was amplified from the chromosomal DNA of *M. maripaludis* S2 with primers with NO3-MM7-F and NO3-MM7-R. The vector was amplified from pMEV4mTs-C-terminal Flag-Strep$_2$ tag plasmid with the phosphate-regulated promoter with BB-NO3-FIRST PART-R and BB-NO3-FIRST PART-F primers. The rest of the procedure is the same as the cloning of SH-10. Sequences of primers can be found in Table 26.

Second step:

The assembled plasmid was linearized with BB-NO3-SECONDPART-F and BB-NO3-SECONDPART-R primers. The *atwA* gene was amplified with A2-NO3-F and A2-NO3-R primers. The rest of the procedure is the same as the cloning of SH-10. Sequences of primers can be found in Table 26.

Table 26) Primers for cloning of plasmid SH-14

Primers names	5´ to 3´
NO3-MM7-F	TAGGGTATACCATAAACCTGGGAGGTGTCTCATATGTACCAGATTATTAGATATGAAG
NO3-MM7-R	TGATCCTTATAATCGCCGTCGTGGTCCTTGTAGTCAGCTTTATTTTCATCCGTTT
BB-NO3-FIRSTPART-F	ATGAGACACCTCCCAGGTTTATG
BB-NO3-FIRSTPART-R	GACTACAAGGACCACGACGG
BB-NO3-SECONDPART-F	ACTAGTAGCGGCCGCTG
BB-NO3-SECONDPART-R	TTACTTTTCGAACTGGGGGTG
A2-NO3-R	ATCCTGCAGCGGCCGCTACTAGTTTAATGGCCGATCATTTCATCTTTT
A2-NO3-F	CTTGGAGCCACCCCCAGTTCGAAAAGTAAATCGCTTAGGTGGTATTAATGTTACT

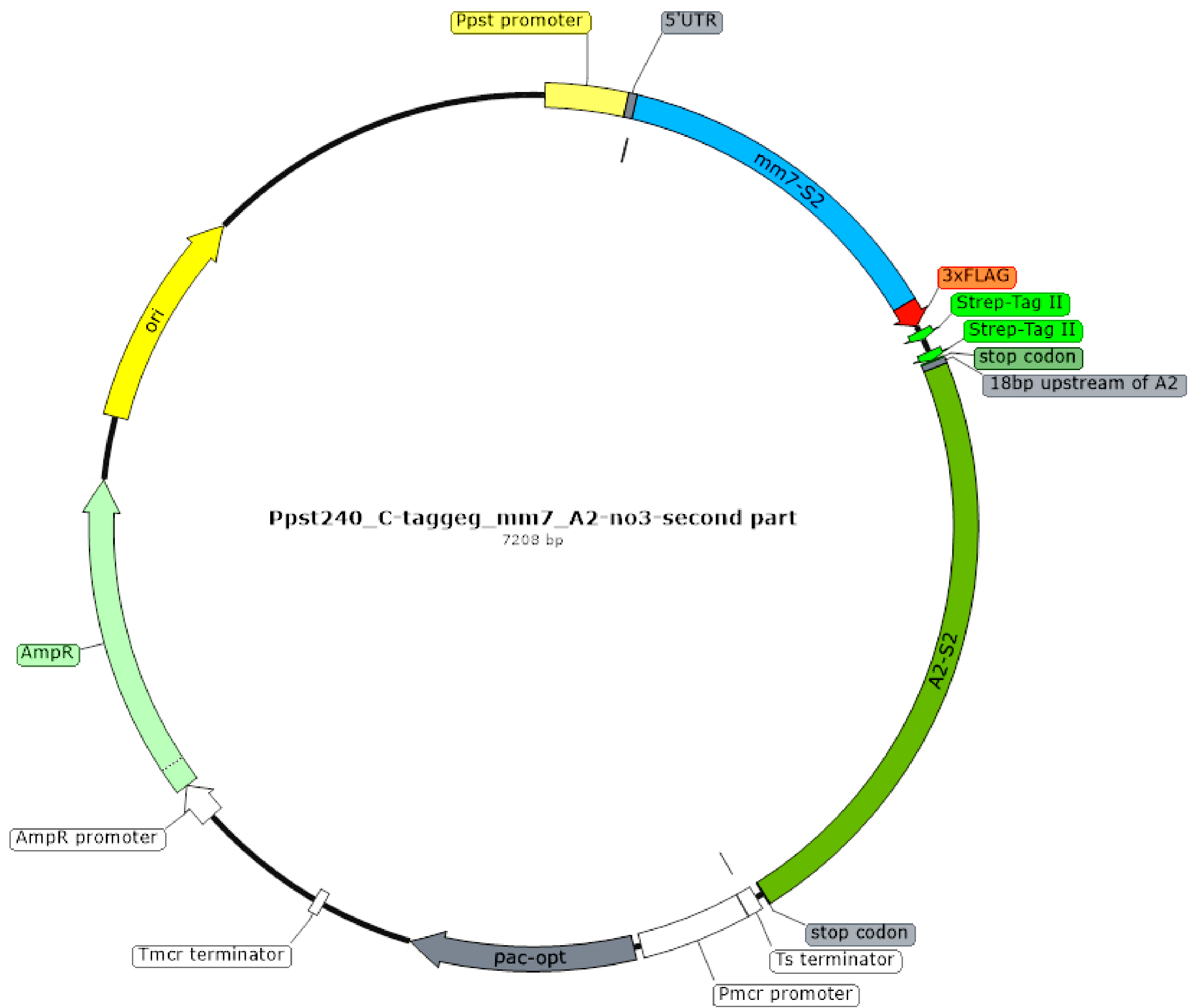

Figure 42) Map of the plasmid SH-14

3.2.2) Transformation (polyethylene glycol mediated)

Buffer and solutions preparation:

Transformation Buffer (TB) (50 mM Tris, 0.35 M sucrose, 0.38 M NaCl, 1 mM $MgCl_2$, 0.00001% rezasurin, pH 7.5)

PEG+TB solution (40% (wt/vol)): PEG8000 (8 g) was added into 10 ml TB buffer and was heated and stirred until dissolved. Then, the volume was adjusted to 20 mL with TB buffer.

Both TB buffer and PEG+TB solution were transferred into the anaerobic chamber and stirred overnight. Cysteine·HCl powder was added to both TB buffer and PEG+TB solution to the final concentration of 5% (wt/vol) and stirred until dissolved. The solutions were transferred to small glass tubes, sealed, pressurized with N_2 gas, and stored at -80 °C. Both buffers have no color and are anaerobic before freezing.

Solid medium 1% (wt/vol) agar FG medium + N*A2*S (2.5% (wt/vol) + puromycin (0.25 mg/ml): Agar (0.3 g), anaerobic FG medium (30 mL), and Na_2S (600 µL, 2.5% (wt/vol)) were added into a 70 mL serum bottle in the anaerobic chamber and sealed. The bottle was pressurized with (4:1, v/v) N_2/CO_2 and then autoclaved on a liquid cycle. The bottle was transferred into an anaerobic chamber. When the temperature dropped to below 50 °C, puromycin (600 µL, 2.5 µg/mL) was added and mixed. The bottle was left at room temperature to cool completely.

Procedure

Fresh-grown *M. maripaludis* S2 culture (10 mL) was centrifuged at 3,900 *g* for 10 min. The supernatant was discarded. TB (500 µL) was added to the pellet and pipetted gently. The mixture was centrifuged at 6,500 *g* for 10 min. The supernatant was discarded and TB+PEG solution (50 µL) was added to the pellet and pipetted gently. The plasmid (0.4-0.6 µg) was added and pipetted gently up and down 10 times. It was transferred into the incubator (37 °C) for 2 hrs, then it was centrifuged for 2 min at 6,500 *g*. The supernatant was discarded, and broth (1 mL) was added and pipetted gently, and it was centrifuged for 2 min at 6,500 *g*. The supernatant was discarded, and broth (1 mL) was added and pipetted gently. The mixture was transferred into the 5 mL broth tube with Na_2S. It was incubated at 37 °C overnight. 1 mL was added to the puromycin plate (solid medium) and incubated for two days to be absorbed by the solid medium. Then it was transferred into the incubator at 37 °C for one week.

3.2.3) Expression and Purification

M. maripaludis cultures expressing recombinant proteins were grown at 37 °C in a 3 L formate medium with 80 µM K_2HPO_4 (low phosphate media) until they reached an absorbance at 600 nm of 0.9. Protein purification was performed under anoxic conditions. The cells were harvested by centrifugation at 7,000 × *g* for 25 min at 4 °C and then resuspended in 25 mL buffer A (100 mM Tris-HCl (pH 7.6), 150 mM NaCl, and Protease Inhibitor Cocktail Tablets (cOmplete™, Mini, EDTA-free, Roche)). Cells were lysed by sonication using four cycles of 5 sec on/off for 3 min and an amplitude of 40% on ice. The cell lysate was centrifuged at 6000 × *g* for 20 min at room temperature. The supernatant fraction was loaded on a gravity column (10 mL) containing 1 mL of Strep-Tactin Superflow Plus resin (IBA Lifesciences, Göttingen, Germany) equilibrated with buffer A. The column was washed several times with buffer A (around 50 mL), and the proteins were eluted with the elution buffer (100 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 2.5 mM desthiobiotin). The eluted fractions were concentrated to a final volume of 50-100 µL with a Nanosep® Centrifugal Device with Omega™ Membrane 10K by centrifugation at 5,000 × *g* for 20 minutes at room temperature and stored at room temperature.

3.2.4) Size-exclusion Chromatography

A purified recombinant protein with strep Flag-Strep$_2$ tag was concentrated anaerobically with Nanosep® Centrifugal Devices with Omega™ Membrane 10K at room temperature up to 500 µL. The concentrated sample was injected into a Superose^{TM} 6 increase 10/300 GL column and purified anaerobically. Buffer A (50 mM Tris, 100 mM NaCl, pH 7.6) was used for purification.

3.2.5) EPR

Continuous wave (CW) EPR spectra were measured at X-band (9 GHz) frequency on a Bruker EMX spectrometer, fitted with the ER-4119-HS high sensitivity perpendicular-mode cavity. The Oxford Instrument ESR 900 flow cryostat in combination with the ITC4 temperature controller was used for measurements in the 4 K to 300 K range using a liquid helium flow.

3.2.6) Mass spectrometry

See section 2.3.8 in chapter 2.

3.3) Results

3.3.1) McrC recombinant protein

The *mcrC* gene was cloned successfully in pMEV4mTs plasmid with N-terminal Flag-$Strep_2$ tag as described in section 3.1.1. The plasmid contains the phosphate-regulated promoter. The plasmid was named SH-10. The transformation of the plasmid into *M. maripaludis* as a host with the polyethylene glycol-mediated method was successful. See section 3.1.2 for more details. The wet cells from a 3 L culture in low phosphate media were around 3.5 g. McrC with Flag-$Strep_2$ was purified anaerobically. The sample did not have color. The sample was run on 10% SDS-Page gel and 10 bands were observed (Figure 43). A band for McrC with strep tag was detected with a western blot experiment (Figure 43). Bands from SDS-PAGE were analyzed with Mass spectrometry finger printing. The results revealed that McrA, McrB, McrG, A2, Mmp17, Mmp7, Mmp3, and McrC proteins were present in the sample. Two bands were identified for McrC. One band is around 27 kDa which is in line with McrC with a strep tag and one at around 21 kDa which is in line for McrC without a strep tag. Table 27 contains information about the proteins present and their molecular weights. Check Tables A3.1 to A3.10 in appendix chapter 3 for the mass spectroscopy data.

To detect the presence of multi-protein complexes the sample was run on a size exclusion column (SEC) (Superose^{TM} 6 increase 10/300 GL column) in the glovebox under the exclusion of air. The FPLC result is shown in Figure 44. Fractions from several peaks were collected and concentrated and run on the SDS-page (Figure 45). Bands from the SDS-PAGE gel was analyzed with MALDI printing mass spectrometry. Table 28 contains information about the proteins present and their molecular weights. McrA, A2, and Heterodisulfide reductase subunit C (HdrC) were present in peak 1 of the SEC. McrA, McrB, McrG, A2, Mmp17, Mmp7, Mmp3, and McrC proteins

were present in peak 2 of SEC. Mmp17, Mmp3, McrC, and Mm17 proteins were present in peak 3 of SEC. Check Table A3.11 to A3.26 in appendix chapter 3 for the mass spectroscopy data. The Size-exclusion FPLC chromatogram was compared to that of the set of molecular weight standards (Figure 44). Peak 2 elution volume is 14.6 mL and peak 3 elution volume is 15.7 ml. The estimated molecular weight of eluted protein samples in Peaks 2 and 3 were 189 and 86 kDa respectively based on a linear fit to the molecular weight standards. Check Figure A3.50 in Appendix Chapter 3 for the Standard curve.

Also, eluted recombinant McrC was run on a native gel and two bands were observed (Figure 46). SDS-PAGE bands were analyzed with MALDI mass spectrometry and Table 29 contains information about the protein present. Both bands contain the same proteins (McrA, McrB, McrG, A2, Mmp17, Mmp7, Mmp3, and McrC proteins). Check Tables A3.27-28 in appendix chapter 3 for the mass spectroscopy data.

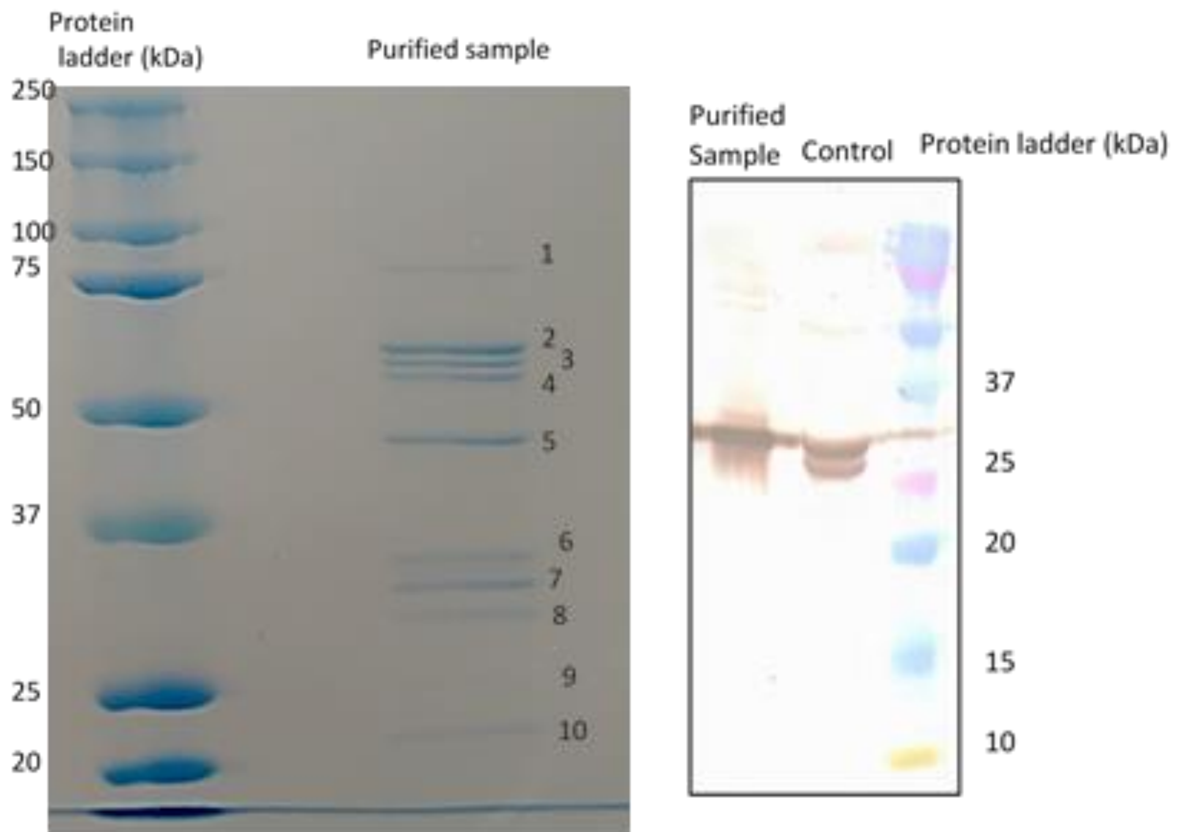

Figure 43) Left panel: McrC protein from *M. maripaludis* was purified anaerobically. Purified McrC protein samples were run on 10% SDS-Page gel. 10 bands were observed. Right panel: western blot results of the purified sample. Bands from SDS-Page were analyzed with Mass spectrometry finger printing.

Table 27) Protein present in the anaerobic McrC purified sample. The raw data is shown in Tables A3.1 to A3.10 in appendix Chapter 3.

SDS-PAGE Band no	MALDI MS	Size (kDa)
1	S-layer protein	58.95
2	McrA	61.08
3	Component A2	59.5
4	Mmp3	56.35
5	McrB	46.65
6	Mmp7	34.97
7	McrG	29.61
8	McrC (+Streptag)	27.59
9	McrC (Native one-no tag)	21.36
10	Mmp17	21.08

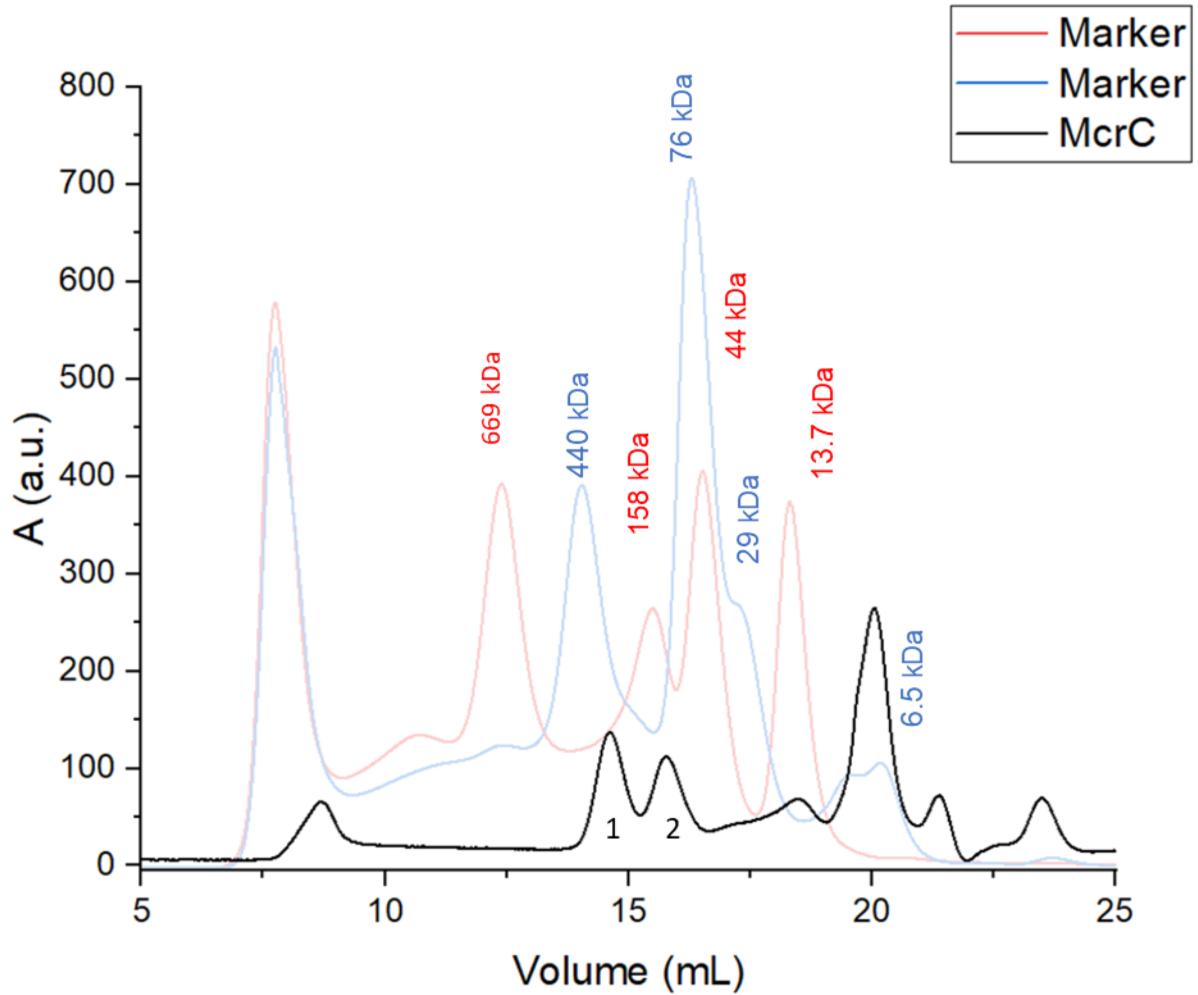

Figure 44) Size exclusion FPLC chromatogram of anaerobically purified McrC. Size-exclusion chromatogram of McrC was compared to the standard proteins (Thyroglobulin (Mr 669 000), Ferritin (Mr 440 000), Aldolase (Mr 158 000), Conalbumin (Mr 75 000), Carbonic anhydrase (Mr 29 000), Ribonuclease A (Mr 13 700) and Aprotinin (Mr 6500)). Check Figure A3.48 in Appendix Chapter 3 for the Standard curve.

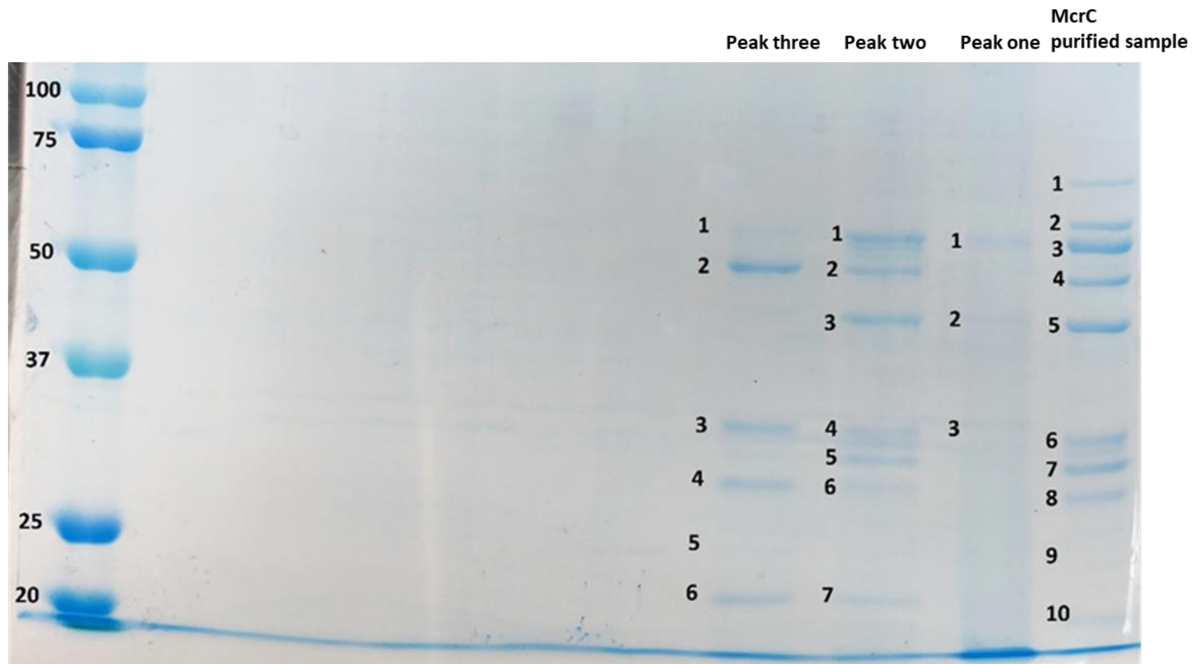

Figure 45) Peaks 1-3 from the size-exclusion profile (Fig. 44) were analyzed by 10% SDS-PAGE. The individual bands were cut-out an analyzed by mass spectrometry.

Table 28) Protein present in the peak 1, 2, and 3 in SEC of anaerobic McrC purified sample. The raw data is shown in Tables A3.11 to A3.26 in appendix Chapter 3.

SDS-PAGE Band no	MALDI MS
Peak 1	
1	McrA Component A2
2	McrB
3	Heterodisulfide reductase subunit C (HdrC)
Peak 2	
1	McrA Methyl coenzyme M reductase, component A2
2	Mmp3
3	McrB
4	Mmp7
5	McrG
6	McrC
7	Mmp17
Peak 3	
1	McrA
2	Mmp3
3	Mmp7
4	McrC with tag F420-dependent methylenetetrahydromethanopterin dehydrogenase (Mtd)
5	McrC
6	Mmp17

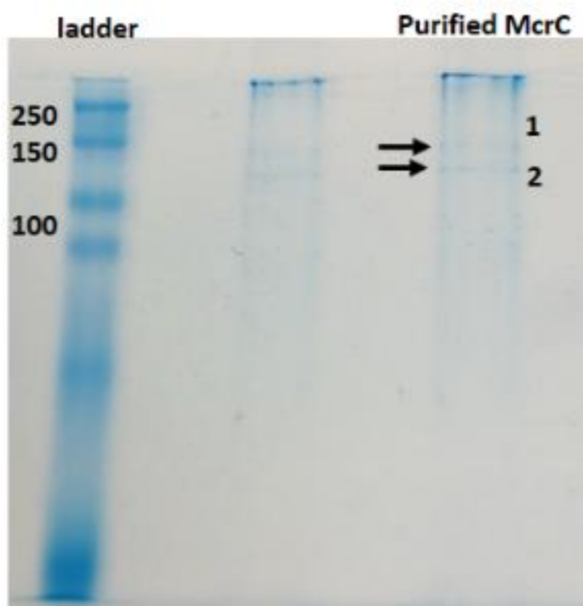

Figure 46) Native page gel of McrC purified sample. The protein ladder used here was for SDS-PAGE gel and not for native gel

Table 29) Proteins were present in two native gel bands of eluted recombinant McrC

Band 1	Band 2
Mmp3	Mmp3
McrC	McrC
Mmp17	Mmp17
Mmp7	Mmp7
McrG	McrG
McrB	McrB
McrA	McrA

3.3.2) Iron-sulfur cluster protein (FFP) recombinant protein

Iron-sulfur cluster protein gene (*FFP*) was successfully cloned in pMEV4mTs with an N-terminal strep tag and approved by DNA sequencing. It was named SH-12. The plasmid contains a phosphate-regulated promoter. The transformation of the plasmid into *M. maripaludis with* polyethylene glycol mediated was successful. The wet cells from a 3 L culture in low phosphate media were around 3 g. The recombinant protein was purified with Strep-Tactin Superflow Plus resin anaerobically and in the glovebox. The sample was run on 10% SDS-PAGE gel and bands were detected (Figure 47). The bands were not analyzed by mass spectrometry. A band for FFP with Strep-tag (27 kDa) was detected with a western blot experiment (Figure 47).

The protein has been renamed several times and it belongs to the family of iron-sulfur flavodoxin proteins. Therefore, it was not unexpected that the protein sample displayed a light brown/yellowish color. The UV-Vis spectrum of the anaerobic purified sample is shown in Figure 48. The spectrum indicates the presence of flavin and possibly iron-sulfur clusters in the sample. The EPR of the sample was measured and the spectrum is shown in Figure 49. The spectrum indicates the presence of a $[4Fe\text{-}4S]^{+}$ cluster in the sample.

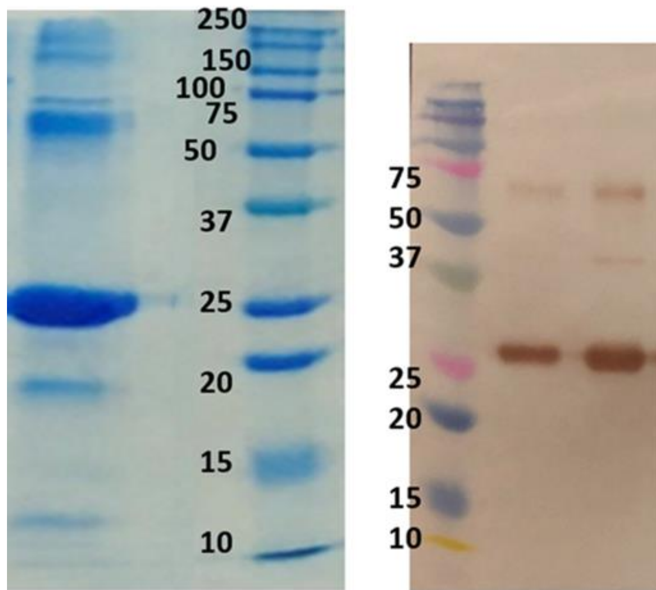

Figure 47) Left one, FFP has purified anaerobically, and protein samples were run on 10% SDS-PAGE gel. Right one: Western blot results of the purified sample. FFP with Strep-tag (27 kDa) was detected.

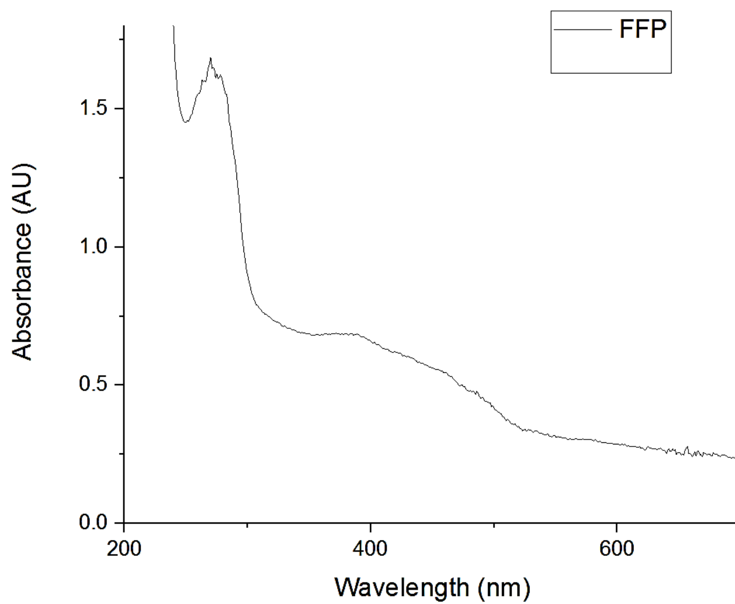

Figure 48) UV-Vis spectrum of FFP protein sample.

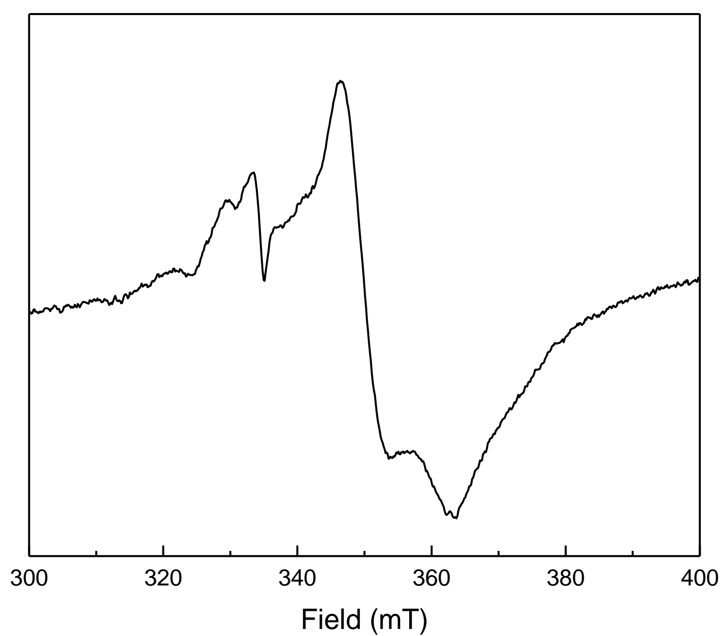

Figure 49) EPR spectra for the FFP protein in the presence of 5 mM dithionite. Measuring conditions: Power, 30 dB; Temperature, 7.9 K; Frequency 9384 MHz.

3.3.3) ATP binding recombinant protein

The ATP-binding protein plasmid was cloned successfully in pMEV4mTs with an N-terminal strep tag and approved by DNA sequencing. The plasmid is called SH-11. The transformation of the plasmid into *M. maripaludis* with polyethylene glycol mediated was successful.

3.3.4) Mmp7 recombinant protein

The plasmid with the *mmp7* gene was cloned successfully in pMEV4mTs with a C-terminal strep tag and approved by DNA sequencing. The plasmid is called SH-14. The transformation of the plasmid into *M. maripaludis* was successful. The wet cells from a 3 L culture in low phosphate media were around 3.5 g. Mmp7 protein with Flag-$Strep_2$ was purified anaerobically in the glovebox. The sample was run on SDS-PAGE and shown in Figure 50. Bands from SDS-PAGE were analyzed with Mass spectrometry finger printing. Table 30 contains information about the proteins present and their molecular weights. McrA, McrB, Mmp3, Mmp7, FrdA, and hydroxymethylglutaryl-coa synthase proteins were present in the sample. Check Tables A3.30 to A3.35 in appendix chapter 3 for raw data Mass spectrometry finger printing.

The purified protein from the affinity column was concentrated and run over a size exclusion column (SEC, Superose^{TM} 6 increase 10/300 GL column) aerobically (Figure 51) and anaerobically (Figure 52). See A3.41 in appendix chapter 3 for more details. It was clear from the FPLC results that anaerobic purification led to the best separation since high-resolution and well-separated peaks were obtained during anaerobic purification. A few peaks were detected in the FPLC result of SEC. Fractions from each peak of SEC were collected and concentrated and run on SDS-PAGE gel (Figure 53). Seven bands were observed for peak number two. McrA, Mmp3, and Mmp7 proteins were present in the peak no: 2 samples. Mmp 3 and McrA proteins were

present in peak no. 3. The raw data Mass spectrometry finger printing is shown in Tables A3.36 to 40 in appendix chapter 3. Size-exclusion chromatogram of Mmp7 was compared to that of a set of molecular weight standards. Peak 2 elution volume is 11.78 ml and peak 3 elution volume is 14.4 ml. The estimated molecular weight of eluted protein samples in Peak 3 was 217 kDa respectively based on a linear fit to the molecular weight standards. Check Figure A3.51 in Appendix Chapter 3 for the Standard curve.

The anaerobic purified Mmp7 protein sample has a brown color. UV–visible spectrum of the sample was measured (Figure 54). The UV-vis spectrum is indicative of the presence of a $[4Fe-4S]^{2+}$ or $[3Fe-4S]^{+}$ cluster, with an absorption band at around 420 nm that loses intensity upon reduction by DIT. Figure 55 shows the EPR spectrum of the sample reduced with dithionite. The spectrum indicates the presence of a $[4Fe-4S]^{+}$ cluster.

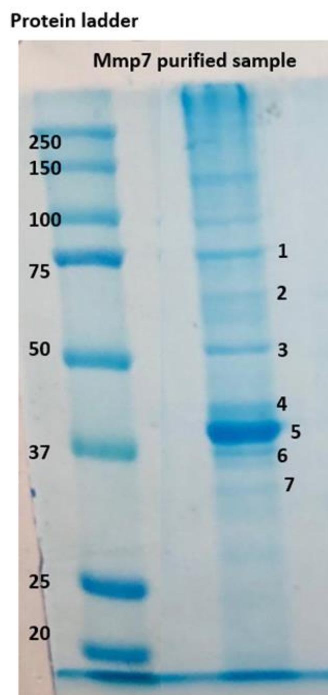

Figure 50) Mmp7 with Flag-Strep2 was purified anaerobically and run in 10% SDS-page gel. The size of Mmp7 with Strep-tag is 41 kDa. Bands from SDS-Page were analyzed with Mass spectrometry finger printing. See table 30 for the assignment. Check Figures A3.29 to A3.35 in appendix chapter 3 for raw data.

Table 30) Protein present in the anaerobic Mmp7 purified sample. The raw data is shown in Figures A3.29 to A3.35 in appendix Chapter 3.

SDS-PAGE Band no	MALDI MS	Size (kDa)
1	CDC48 cell division cycle protein family member	87
2	McrA	61
3	Mmp3	56
4	McrB	47
5	Mmp7	41
6	Hydroxymethylglutaryl-coa synthase	38
7	M. voltae Fumarate reductase/succinate dehydrogenase flavoprotein domain protein (FrdA) (GN=Mvol_0038)	69

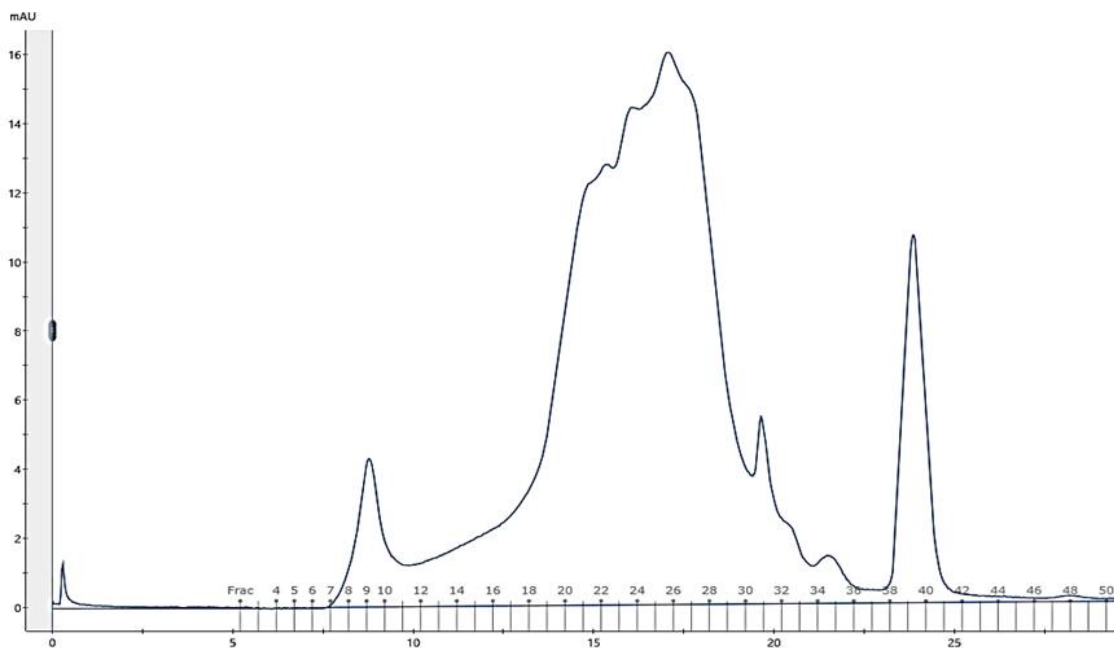

Figure 51) Elution profile for Mmp7 on the SuperoseTM 6 increase 10/300 GL. The sample was run under the exclusion of oxygen.

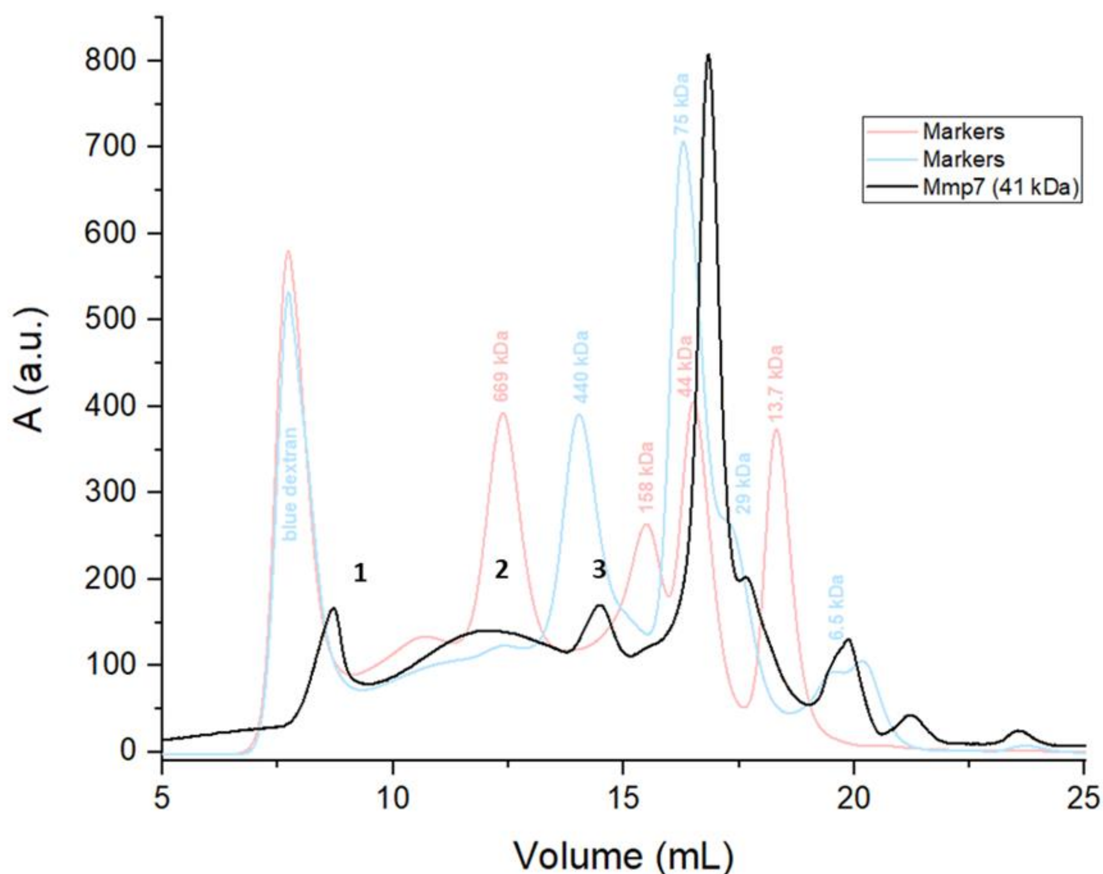

Figure 52) FPLC Size-exclusion chromatogram of Mmp7 Size-exclusion chromatogram of Mmp7 was compared to the standard proteins (Thyroglobulin (Mr 669 000), Ferritin (Mr 440 000), Aldolase (Mr 158 000), Conalbumin (Mr 75 000), Carbonic anhydrase (Mr 29 000), Ribonuclease A (Mr 13 700) and Aprotinin (Mr 6500)). Check Figure A3.49 in Appendix Chapter 3 for the Standard curve.

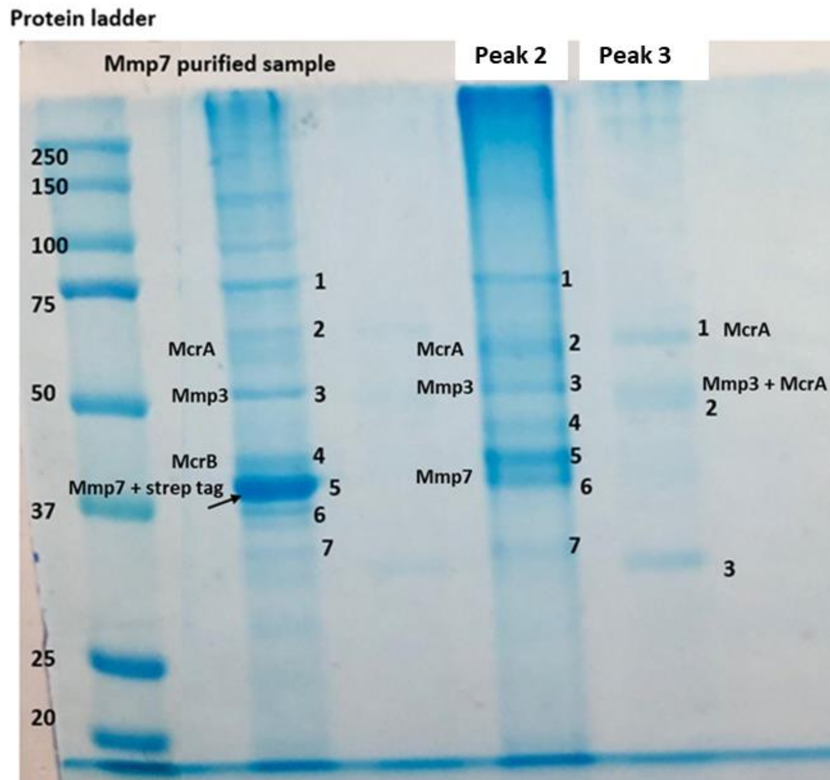

Figure 53) Each peak from SEC was concentrated and run on 10% SDS-Page gel.

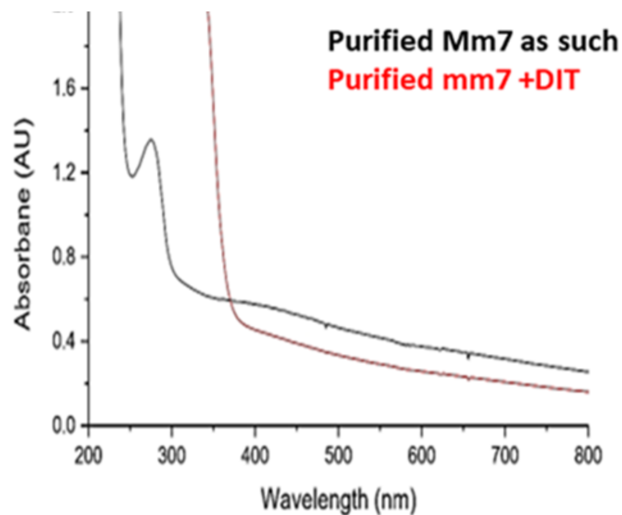

Figure 54) UV–visible spectrum of eluted Mmp7 protein sample.

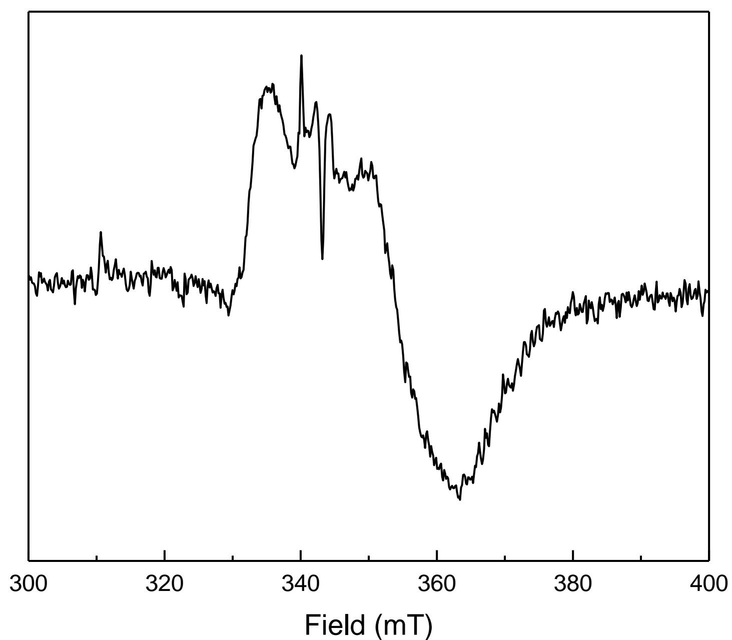

Figure 55) EPR spectrum of the Mmp7 protein sample in the presence of 5 mM dithionite. Measuring conditions: Power, 20 dB; Temperature 8.0 K; Frequency 9384 MHz.

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3.3.5) A2 recombinant protein

The *atwA* gene, with a c-terminal Flag-$Strep_2$ tag, and the *mmp7* gene were cloned in the pMEV4mTs plasmid and approved by DNA sequencing. Both genes share a phosphate-regulated promoter. The plasmid was named SH-13. The transformation of the plasmid into *M. maripaludis* with the polyethylene glycol-mediated method was successful. The wet cells from a 3 L culture in low phosphate media were around 3.5 g. A2 protein with Flag-$Strep_2$ was purified anaerobically in the glovebox. This purification was repeated several times and sometimes the sample showed a weak color. EPR measurements for the colored samples showed the presence of a [4Fe-4S] cluster (Figure 59). The sample was run on SDS-PAGE (Figure 56). Multiple bands were observed. The size of the A2 component protein with Flag-Strep2 is 65.45 kDa. Bands from SDS-PAGE were analyzed with Mass spectrometry finger printing. McrA, McrB, and McrG proteins were present in the sample. The rest of the bands were identified as A2 protein due to degradation of the A2 protein. Check Tables A3.42 to A3.47 in appendix Chapter 3 for raw data Mass spectrometry finger printing.

The samples were subsequently run over a size exclusion column (Superose 6 increase 10/300 GL) in the glovebox. FPLC results are shown in Figure 57 (see Figure A3. 48 in appendix Chapter 3 for more details). Six peaks were detected. Fractions for each peak were collected and concentrated and run in SDS-PAGE (Figure 58). Bands from the SDS-PAGE gel were analyzed with Mass spectrometry finger printing. McrG and A2 proteins were present in the peak 1 sample. Peak 2, 3, and 4 samples only contained A2 protein. Check Table A3.49 in appendix Chapter 3 for the raw data Mass spectrometry finger printing. The Size-exclusion chromatogram of A2 was compared to that of a set of molecular weight standards (Figure 57). Elution volumes of peaks 2, 3, and 4 are 11.6, 13.3, and 16.7 mL respectively. The estimated molecular weight of eluted protein

samples in Peaks 3 and 4 were 476, and 42 kDa respectively, based on a linear fit to the molecular weight standards. Check Figure A3.52 in Appendix Chapter 3 for the Standard curve.

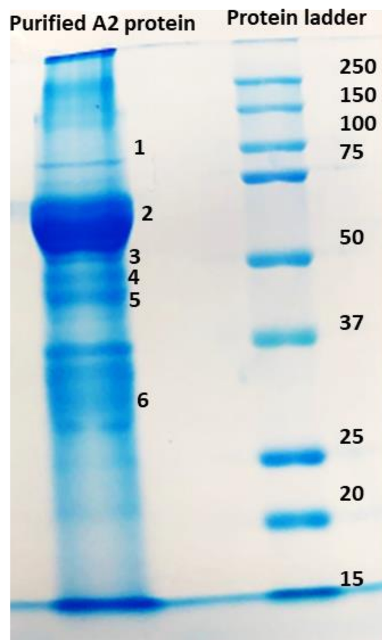


Figure 56) A2 component protein with Flag-Strep2 was purified anaerobically and run in 10% SDS-page gel. The size of component A2 with a strep tag is 65.45 kDa. Bands from SDS-PAGE were analyzed with Mass spectrometry finger printing. A2 protein was present in bands 2 and 4. McrA was present in band 3. McrB was present in band 5. McrG was present in band 6.

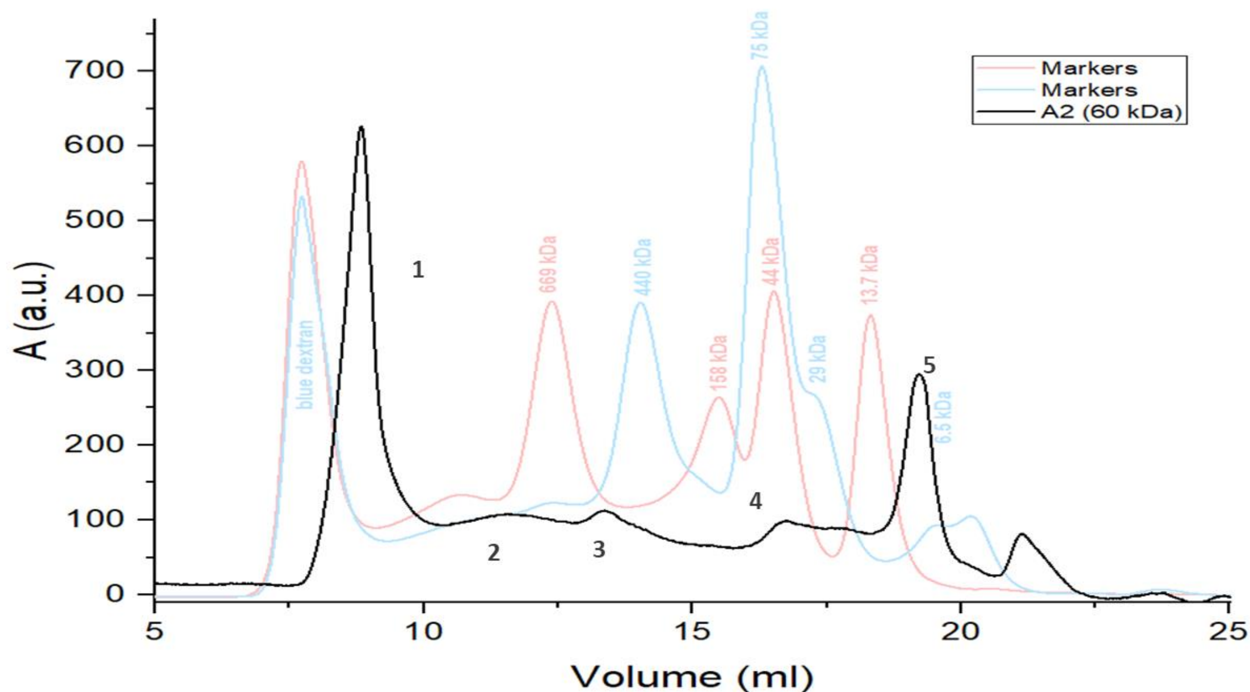


Figure 57) Size-exclusion chromatogram of A2. Size-exclusion chromatogram of A2 protein was compared to the standard proteins (Thyroglobulin (Mr 669 000), Ferritin (Mr 440 000), Aldolase (Mr 158 000), Conalbumin (Mr 75 000), Carbonic anhydrase (Mr 29 000), Ribonuclease A (Mr 13 700) and Aprotinin (Mr 6500)). Check Figure A3.50 in Appendix Chapter 3 for the Standard curve.

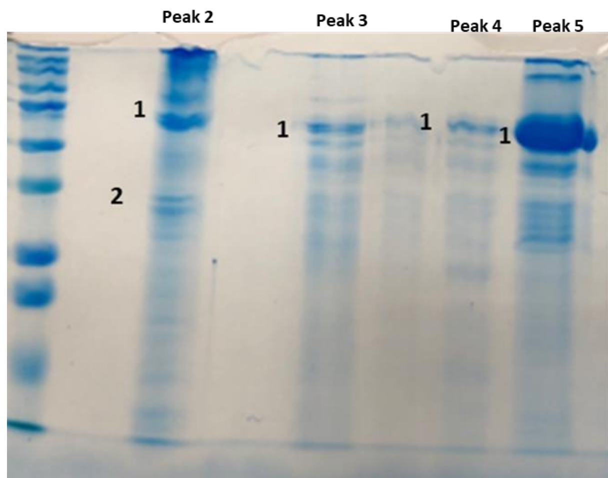


Figure 58) Each peak from SEC of A2 was concentrated and run on 10% SDS-PAGE gel. Although six peaks were detected in SEC only four peaks contain proteins. Bands from SDS-PAGE were analyzed with Mass spectrometry fingerprinting. McrG and A2 protein were present in peak 2. Peak 3, 4 and 5 samples only contained A2 protein. Check Figure A3.46 in appendix Chapter 3 for raw data.

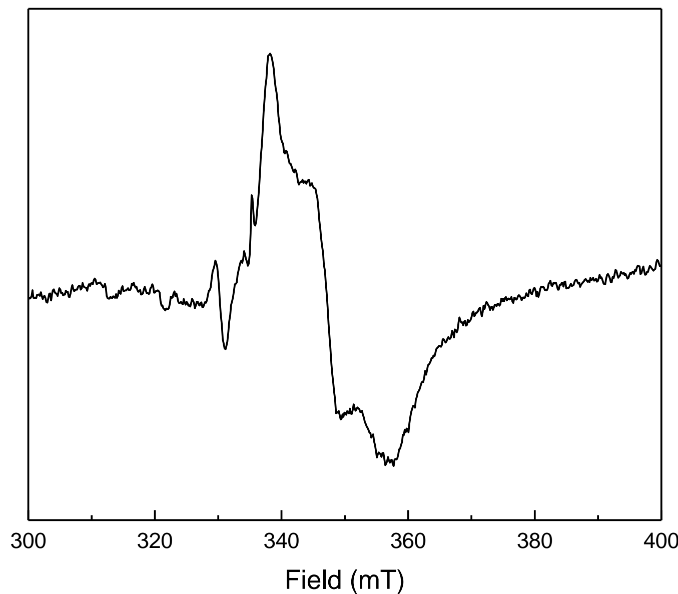


Figure 59) EPR spectrum of the A2 protein sample in the presence of 5 mM dithionite. Measuring conditions: Power, 20 dB; Temperature 8.0 K; Frequency 9384 MHz.

3.4)

3.5) Discussion

A pull-down approach was used to identify protein-protein interactions and reveal the composition of protein complex A3a. Mmp7, McrC, FFP, ATP, and component A2 components were homologously expressed in *M. maripaludis*. Results of Mass spectrometry fingerprinting analysis showed that component A2 interacts with McrA, McrB, and McrG. An additional purification step on a size-exclusion column indicates that A2 might only directly interact with McrG. Using the calibration curve for the column it was shown that one of the peaks is from a complex with a mass of about 476 kDa. Since only A2 was detected in this peak by mass spectroscopy it indicates that the homologously expressed A2 (60 kDa) can form a multimeric complex. EPR measurements showed the presence of [4Fe-4S] clusters. As previously found in expression experiments in *E. coli* the A2 protein is highly sensitive to degradation. A series of A2 bands can be detected on SPS-PAGE with smaller sizes.

Mass spectrometry fingerprinting analysis showed that recombinant McrC eluted together with McrABG, Mmp3, Mmp7, Mmp17, and component A2. The size-exclusion column experiment showed that several complexes are present with variable composition. Analysis of one peak showed the presence of McrABG, Mmp3, Mmp7, Mmp17, McrC, and component A2 with an estimated size of 198 kDa. A second peak contains Mmp7, Mmp3, McrC, and Mmp17 with an estimated size of 86 kDa. If the ratio of McrA:McrB:McrG:Mmp3:Mmp7:Mmp17:(component A2):McrC was 1:1:1:1:1:1:1:1, the size of the complex would be around 336 kDa. If the ratio of Mmp3:Mmp7:Mmp17:McrC was 1:1:1:1:1, the size of the smaller complex would be around 140 kDa. Some subunits must be present in smaller quantities. Additional experiments will be needed to determine the ratio of the individual subunits in this complex. The Agilent 2100 bioanalyzer

present in the teaching laboratory would be able to provide that data. Similarly, two bands were detected in the native gel indicative of the presence of two complexes with different compositions.

Mmp7 co-purified with McrA, Mmp3, and McrB on the Strep-tag column. Further separation on the size-exclusion column resulted in two fractions pointing to the presence of two protein complexes in the sample. The estimated size for the top of peak 3 is 216 kDa. McrA, Mmp3, Mmp7, and McrB were present in peak no 2. Peak 2 is larger than 600 kDa, about 700-800 kDa. McrA and Mmp3 were present in peak no 3.

This project is a collaboration with the group of Dr. Whitman at the University of Georgia. Based on our data they also expressed the MrcC, FFP/Isp, Mmp3, and Mmp17 in *M. maripaludis* and had the samples characterized via mass spectrometry. The collective data from both groups are summarized in table 31. The first column shows the proteins that were expressed. The other columns showed the presence of the pulled-down proteins. Dark blue means a high Mascot score and light blue a low score. The shades in between indicate medium scores.

Table 31) Protein present in the purified sample of Mmp3, Mmp17, A2, Mmp7, and McrC

Proteins Expressed	**Proteins 'pulled down'**											
	McrABG	**McrC**	**McrC-tag**	**A2**	**Mmp3**	**Mmp7**	**Mmp17**	**Hdr**	**Vhu**	**Fdh**	**Fwd**	**Cdh**
McrC												
Mmp7												
A2												
Mmp3												
Mmp17												
Isp												

From this data, it can be concluded that *M. maripaludis* contains a protein complex that contains McrC, Mmp3, Mmp7, and Mmp17. The whole complex interacts directly with Mcr. A2 does not appear to interact with this complex but directly with Mcr (McrG). Iron-sulfur cluster protein (FFP/Isp) and ATP binding protein do not appear to be part of this complex. Most of the proteins appear to be in contact with several of the other components. Figure 60 shows a simplistic scheme of what the A2: Mcr: Mmp-Cluster complex could look like.

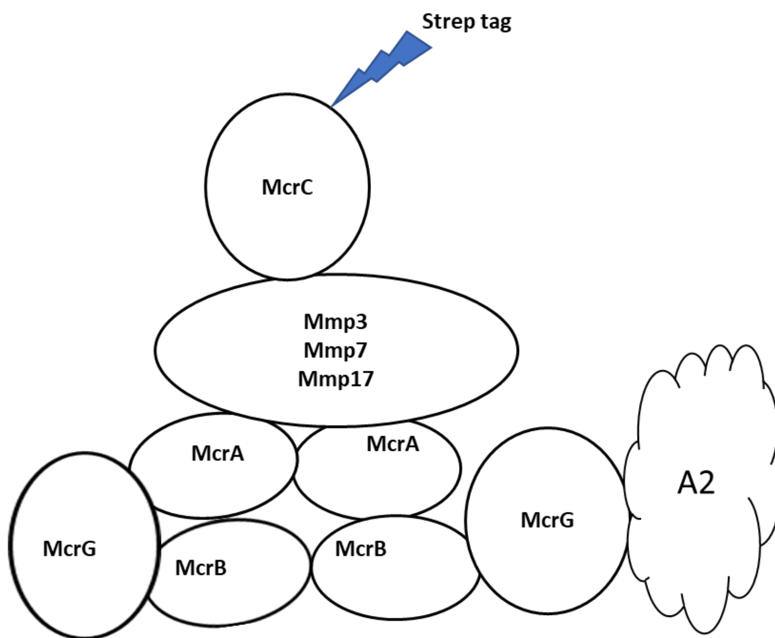


Figure 60) Proposed protein complex for McrC from M. maripaludis S2

Chapter 4 : Summary

This study aimed to investigate and identify the A3a complex. We present evidence here for a complex in *M. maripaludis* that contains McrC, Mmp3, Mmp7, and Mmp17. This complex appears to directly interact with Mcr. The A2 protein does not appear to interact with this complex but directly with Mcr.

The second aim was to investigate whether the *mmp* cluster from *M. acetivorans* encodes a large complex. Based on this study, 7 proteins, A2, Mmp2, 3, 5, 6, 7, 15, and 17 form a complex. Mmp3 was absent when the complex was exposed to air during purification. It can be assumed that these proteins have their native three-dimensional structure in *E. coli* since a full complex was assembled. It is probably the first time that 8 genes from archaea with 8 promoters were cloned together in one plasmid and were successfully expressed in *E. coli*. The *mmp2* gene is not part of the *mmp* cluster but it did show up in the expressed complex.

A comparison of both sets of data shows a discrepancy between the Mmp complex in *M. acetivorans* and *M. maripaludis*. Although we expect that both should have similar Mmp clusters this does not have to be the case. From previous studies, we know that *M. marburgensis* and *M. maripaludis* contain a soluble Hdr/hydrogenase complex that is capable of electron bifurcation and appears to be important for the activation of Mcr. *M. acetivorans* does not appear to have such a protein. Its Hdr complex is membrane-bound and does not bifurcate. Still, it would be important to homologously express the *mmp2*, *5*, *6,* and *15* genes of *M. maripaludis* to rule out that they are not part of the complex. In addition, we need to add a 9th gene to the plasmid that of McrC since it appears to be part of the *mmp* cluster.

Although it was expected that some of the proteins that were initially assigned to the activating complex (Chapter 1, table 3) might not be part of it, it was still unexpected that the ATP-

binding protein was not part of it. The FFP/Isp was included because of the presence of the flavin and [4Fe-4S] clusters but the redox potential would have been too high to be able to reduce Mcr. Since the Mmp complex and some of the individual subunits can bind 4Fe clusters the FFP/Isp protein would in principle be unnecessary.

Iron-sulfur clusters are most likely important for the function of this protein since there would be a need for electron transfer. Most likely the Mmp cluster expressed in *E. coli* will not have a full complement of cofactors. However, the absorption date showed a small change in the intensity of the 420 nm band upon the addition of dithionite. It is possible that the dithionite is not strong enough to reduce the clusters in this complex resulting in a very weak EPR signal (Figure 32). This experiment should be repeated with other reductants with lower redox potential.

In an alternative approach, the Mmp clusters could be tagged directly in the *M. maripaludis* genome for easy and quick purification. In case the expression levels are low, which they probably are based on the work with *M. marburgensis*, it should be attempted to overexpress all subunits as was done here in *E. coli*.

A2 may not be part of the Mmp complex protein in *M. acetivorans* too. Size exclusion chromatography of the Mmp cluster protein sample would answer this question. The result would show if it is possible to get separate peaks for the A2 protein and the Mmp cluster.

In figure 60 we presented a model of the McrC/Mmp/Mcr complex. Dr. Mansoorabadi went ahead and used that basic model to simulate a full-scale model using AlphaFold. In this model, the Mmp cluster is a dimer and interacts with two Mcr. The idea for the dimer came from the dimer structure for Mmp15. A dimer of Mmp15 connects both monomers and could coordinate up to 3 iron-sulfur clusters between both subunits (Figure 61).

The model shows A2 interacting with Mcr. In the model, he included a Zn^{2+}. Although four cysteine residues are present in a $CxxCx_{12}CxxC$ motive. Dr. Mansoorabadi thought the Cys were placed too close to coordinate 4Fe clusters (Figure 62).

This model shows Mmp7 interacting with McrC. Mmp7 only has 2 Cys residues and needs to form a dimer to be able to coordinate a 4Fe cluster (Figure 63). In the model, we propose that a cluster is present in between the Mmp and McrC subunits.

The Mmp3, Mmp7, Mmp17, and McrC subunits have interactions with each other and with Mcr. The whole model space-filling and the ribbon representation are shown in Figures 64 and 65. We will need to purify this complex and obtain the structure and see how much of these predictions are correct.

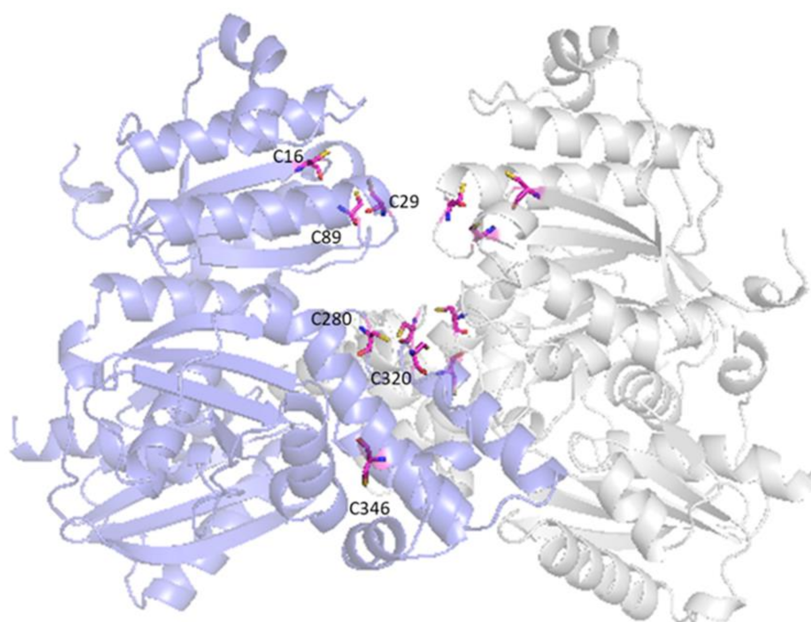


Figure 61) Predicted structure of a hypothetical dimer of Mmp15. There are some cysteines, and they could make clusters within the dimer. The structures were calculated with the online AlphaFold server by Dr. Steven Mansoorabadi.

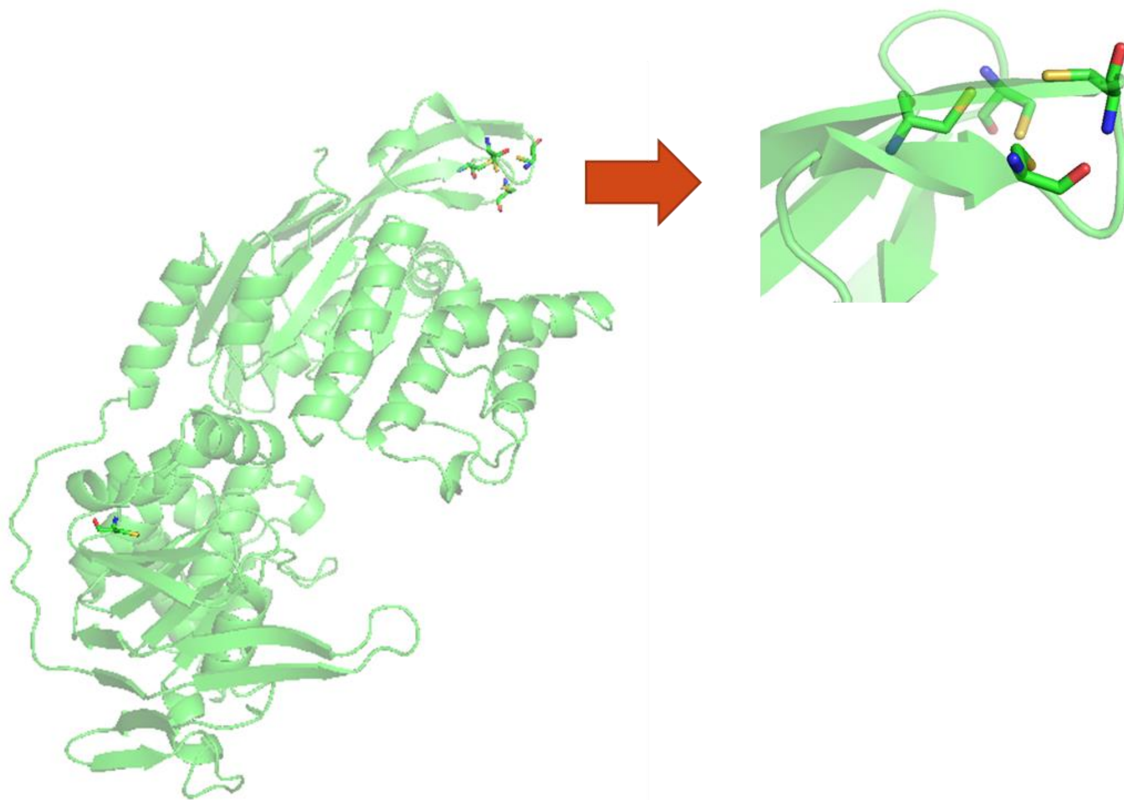


Figure 62) Predicted structure of A2 component from *M. acetivorans*. A2 has four cysteines (C70, C73, C86, and C89) that could bind a Zn (II) or divalent metal ion such as Fe (II). Four cysteine residues are present in a CxxCx12CxxC motive.

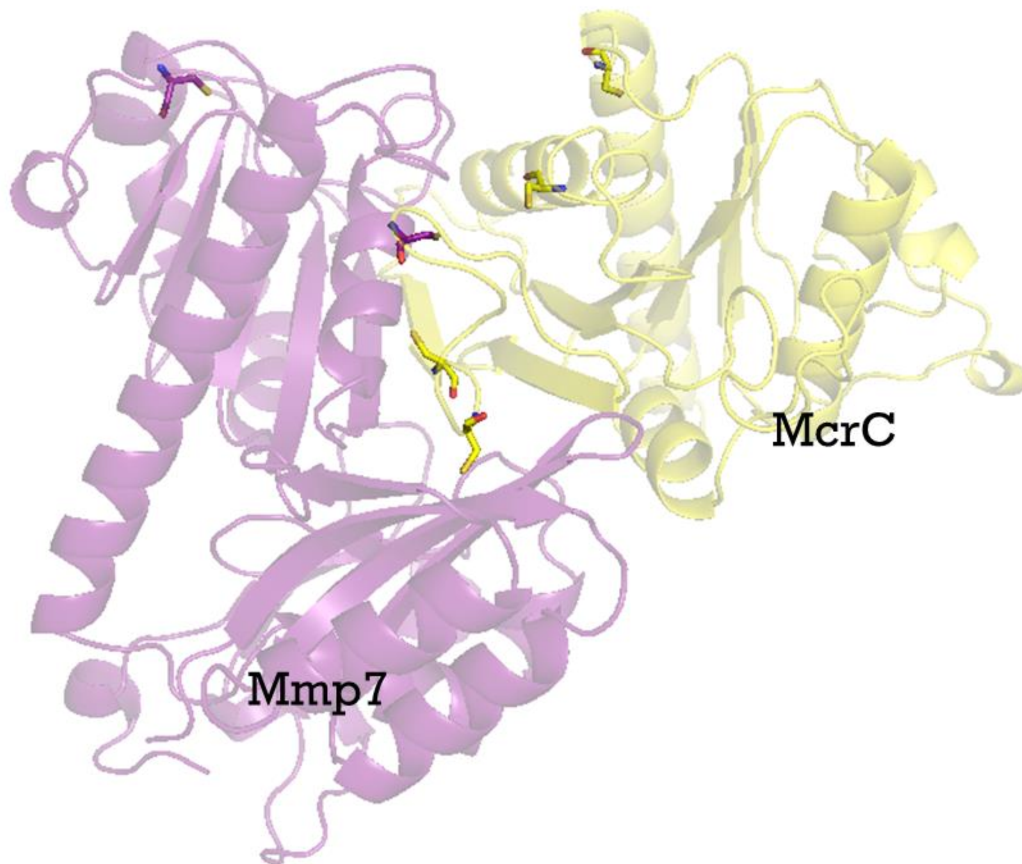


Figure 63) The Mmp7 protein has conserved cysteine residues and a hypothetical dimer with McrC could potentially coordinate an iron-sulfur cluster. The yellow color presents McrC and the purple color presents Mmp7.

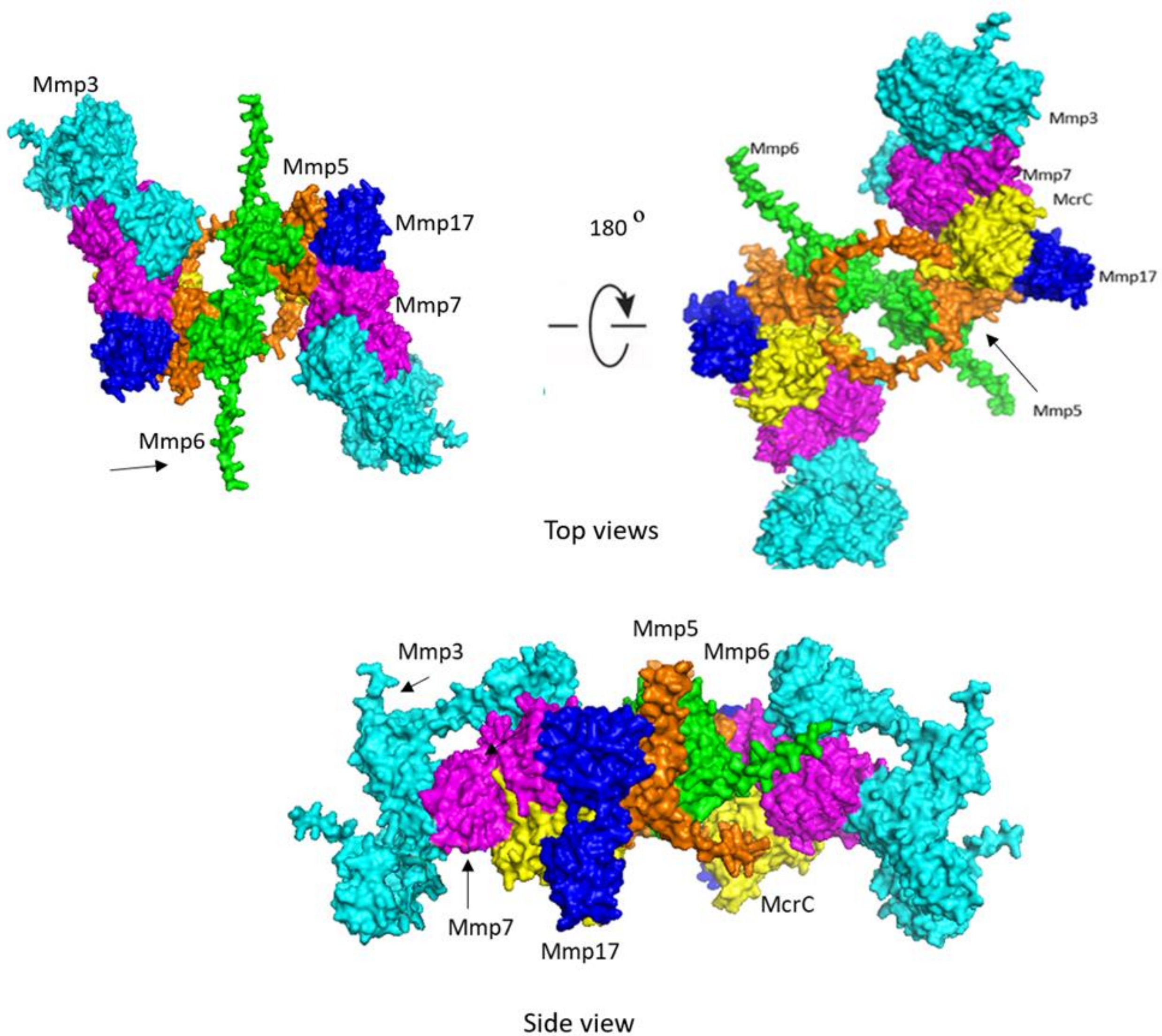


Figure 64) Three views of the predicted protein complex structure of Mmp7, Mmp17, McrC, Mmp6, Mmp3, and Mmp5 from *M. acetivorans*.

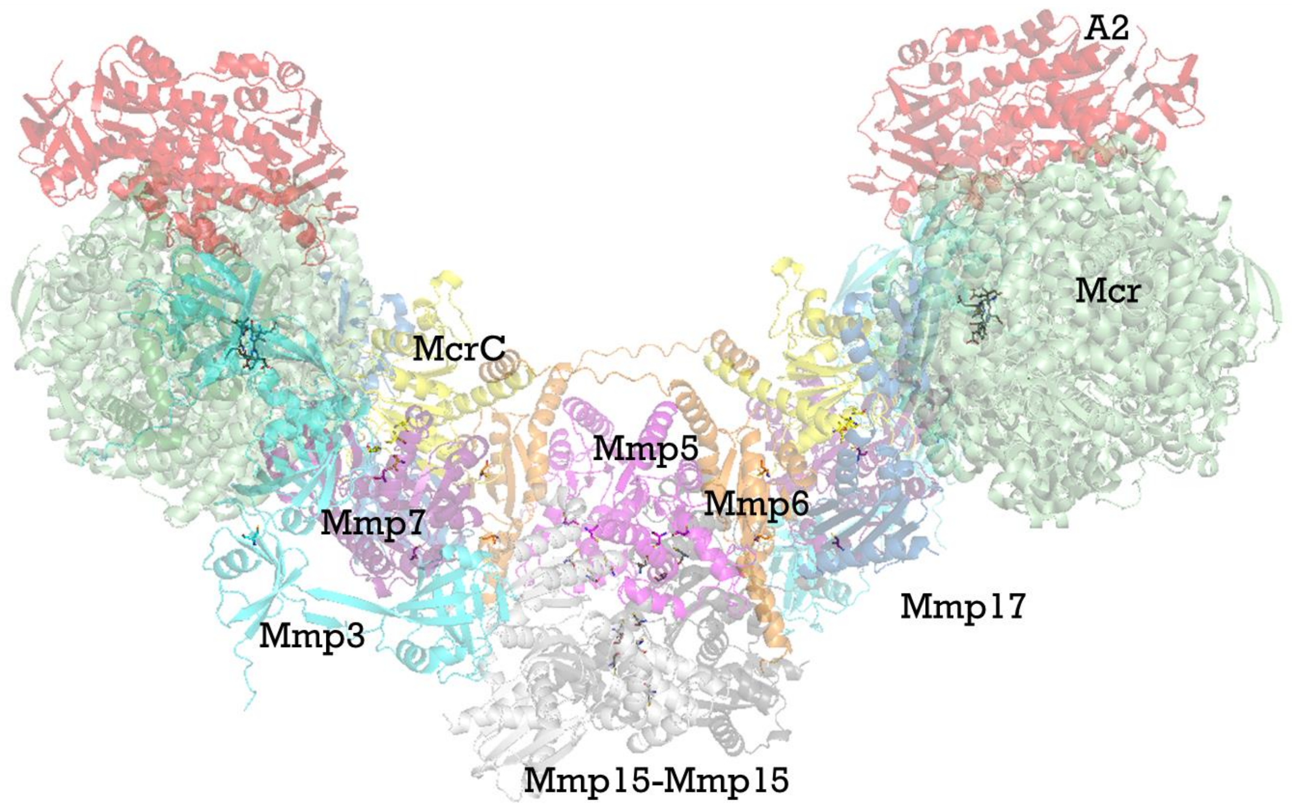


Figure 65) The predicted complex structure of A2: Mcr: Mmp-Cluster (*M*. *acetivorans*).

Appendix for Chapter 2

A2. 1)Raw data of Mass spectrometry analysis of the complete purified Mmp sample (Aerobic purification)

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
AAM07348.1	ABC transporter, ATP-binding protein [*Methanosarcina acetivorans* C2A]	20366.42	74.30	1	41	41	654	537	59.6	5.58
AAM07344.1	BadF/BadG/BcrA/BcrD ATPase [*Methanosarcina acetivorans* C2A]	6464.02	61.45	1	21	21	147	415	44.0	5.25
AAM07342.1	conserved hypothetical protein [*Methanosarcina acetivorans* C2A]	677.45	53.05	1	15	15	21	311	34.3	6.47
AAM07345.1	conserved hypothetical protein [*Methanosarcina acetivorans* C2A]	596.99	63.53	1	10	10	22	170	19.0	4.88
AAM07346.1	conserved hypothetical protein [*Methanosarcina acetivorans* C2A]	507.98	48.73	1	7	7	13	158	17.8	6.37
AAM07539.1	AIR synthase related protein [*Methanosarcina acetivorans* C2A]	268.87	31.33	1	10	10	10	332	35.6	4.68
AAM07343.1	conserved hypothetical protein [*Methanosarcina acetivorans* C2A]	128.44	37.19	1	6	6	6	199	22.7	5.26
AAM07347.1	conserved hypothetical protein [*Methanosarcina acetivorans* C2A]	77.59	4.00	1	2	2	2	525	58.3	4.92

A2. 2) Raw data for Mass spectrometry analysis of the complete purified Mmp sample (Anaerobic purification)

Description	Coverage [%]	# Peptides	# PSMs	# Unique Peptides	MW [kDa]	Score	# Peptides	Gene ID
methanogenesis marker 15 protein [*Methanosarcina acetivorans*]	98	42	153	40	44	307.54	42	A0A832S8W1; MA_3994; Q8TIZ5
submitted name: Glycine betaine/carnitine/choline transport ATP-binding protein OpuCA [*Methanococcus maripaludis*]	85	48	175	43	59.5	328.11	48	A0A2L1C9A1; MMJJ_03630
submitted name: ATPase [Methanococcus maripaludis OS7*]*	69	22	47	20	43.9	81.37	22	A0A2Z5PJN6; MMOS7_06520
methanogenesis marker 5 protein [*Methanosarcina acetivorans* C2A]	56	10	20	10	19	25.02	10	MA_3995; Q8TIZ4
methanogenesis marker 3 protein [*Methanosarcina acetivorans* C2A]	32	14	33	14	58.3	64.17	14	A0A832VXF9; MA_3997; Q8TIZ2
methanogenesis marker 7 protein [*Methanosarcina acetivorans*]	28	4	6	4	34.3	7.81	4	A0A832SF80; MA_3992; Q8TIZ7
methyl coenzyme M reductase system, component A2 [*Methanosarcina acetivorans*]	16	7	14	6	59.6	21.05	7	A0A832W783; MA_3998; Q8TIZ1
methanogenesis marker 2 protein [*Methanosarcina acetivorans*]	14	4	5	4	35.6	4.05	4	A0A832VXH7 MA_4193; Q8TIF9

Appendix for Chapter 3

A3. 1) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 1 from purified McrC sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Q6M088|Q6M088_METMP Mass: 58912 Score: 154 Expect: 1.6e-011 Matches: 16 S-layer protein OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=slp PE=4 SV=1 A0A7J9PWD1|A0A7J9PWD1_METMI Mass: 58382 Score: 35 Expect: 13 Matches: 6 S-layer protein (TIGR01564 family) OS=Methanococcus maripaludis OX=39152 GN=HNP95_001209 PE=4 SV=1 A9A7I1|A9A7I1_METM6 Mass: 13832 Score: 34 Expect: 15 Matches: 4 Transcriptional regulator PadR family protein OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=MmarC6_0105 PE=4 SV=1 A6UTE7|A6UTE7_META3 Mass: 19469 Score: 34 Expect: 18 Matches: 4 Probable Brix domain-containing ribosomal biogenesis protein OS=Methanococcus aeolicus (strain ATCC BAA-1280 / DSM 17508 / OCM 812 / Nankai-3) OX=419665 GN=Maeo_0177 PE=3 SV=1

A3. 2) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 2 from purified McrC sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 241 Expect: 3.1e-020 Matches: 30 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1 A0A7J9NQV7|A0A7J9NQV7_METMI Mass: 61031 Score: 241 Expect: 3.1e-020 Matches: 30 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP86_000167 PE=3 SV=1 G0H3B3|G0H3B3_METMI Mass: 61040 Score: 228 Expect: 6.3e-019 Matches: 28 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08645 PE=3 SV=1 A0A2L1CBB0|A0A2L1CBB0_METMI Mass: 61038 Score: 228 Expect: 6.3e-019 Matches: 28 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=mcrA PE=3 SV=1 A0A2Z5PTJ7|A0A2Z5PTJ7_METMI Mass: 61024 Score: 228 Expect: 6.3e-019 Matches: 28 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrA PE=3 SV=1 A0A7J9PKB0|A0A7J9PKB0_METMI Mass: 61040 Score: 228 Expect: 6.3e-019 Matches: 28 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP93_000168 PE=3 SV=1 A0A7J9S9S4|A0A7J9S9S4_METMI Mass: 61024 Score: 228 Expect: 6.3e-019 Matches: 28 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP96_000849 PE=3 SV=1 A0A2Z5PKS6|A0A2Z5PKS6_METMI Mass: 61016 Score: 216 Expect: 9.9e-018 Matches: 27 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrA PE=3 SV=1 H7CHX3|H7CHX3_METMI Mass: 41475 Score: 108 Expect: 6.3e-007 Matches: 15 Coenzyme-B sulfoethylthiotransferase (Fragment) OS=Methanococcus maripaludis OX=39152 GN=mcrA PE=3 SV=1 H7CHX4|H7CHX4_METMI Mass: 40899 Score: 88 Expect: 6e-005 Matches: 13 Coenzyme-B sulfoethylthiotransferase (Fragment) OS=Methanococcus maripaludis OX=39152 GN=mcrA PE=3 SV=1
2	A0A7J9NYX4|A0A7J9NYX4_METMI Mass: 60983 Score: 227 Expect: 7.9e-019 Matches: 28 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP87_000167 PE=3 SV=1

	A0A7J9S5U7|A0A7J9S5U7_METMI Mass: 61031 Score: 214 Expect: 1.6e-017 Matches: 27 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP92_000701 PE=3 SV=1
3	A4FVW6|A4FVW6_METM5 Mass: 61055 Score: 188 Expect: 6.3e-015 Matches: 26 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0017 PE=3 SV=1
4	A6VHE7|A6VHE7_METM7 Mass: 61100 Score: 176 Expect: 9.9e-014 Matches: 25 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_0806 PE=3 SV=1 A0A7J9PJ28|A0A7J9PJ28_METMI Mass: 61100 Score: 176 Expect: 9.9e-014 Matches: 25 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP90_000389 PE=3 SV=1 A9A9A2|A9A9A2_METM6 Mass: 61112 Score: 139 Expect: 5e-010 Matches: 22 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=MmarC6_1111 PE=3 SV=1
5	P07961|MCRA_METVA Mass: 60799 Score: 110 Expect: 3.9e-007 Matches: 21 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus vannielii OX=2187 GN=mcrA PE=3 SV=1
6	A6UQK5|A6UQK5_METVS Mass: 60771 Score: 110 Expect: 3.9e-007 Matches: 21 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus vannielii (strain ATCC 35089 / DSM 1224 / JCM 13029 / OCM 148 / SB) OX=406327 GN=Mevan_0872 PE=3 SV=
7	D7DUH5|D7DUH5_METV3 Mass: 61260 Score: 77 Expect: 0.00077 Matches: 18 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus voltae (strain ATCC BAA-1334 / A3) OX=456320 GN=Mvol_1128 PE=3 SV=1 P11559|MCRA_METVO Mass: 61188 Score: 77 Expect: 0.00079 Matches: 17 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus voltae OX=2188 GN=mcrA PE=3 SV=1

A3. 3) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 3 from purified McrC sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 272 Expect: 2.5e-023 Matches: 33 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1 A0A2Z5PV80|A0A2Z5PV80_METMI Mass: 59453 Score: 272 Expect: 2.5e-023 Matches: 33 Methyl coenzyme M reductase system component A2 OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_06650 PE=4 SV=1 G0H4C2|G0H4C2_METMI Mass: 59453 Score: 272 Expect: 2.5e-023 Matches: 33 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_03370 PE=4 SV=1 A0A7J9NUM7|A0A7J9NUM7_METMI Mass: 59440 Score: 272 Expect: 2.5e-023 Matches: 33 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP86_001138 PE=4 SV=1 A0A7J9RZV0|A0A7J9RZV0_METMI Mass: 59426 Score: 272 Expect: 2.5e-023 Matches: 33 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001226 PE=4 SV=1 A0A2L1C9A1|A0A2L1C9A1_METMI Mass: 59453 Score: 272 Expect: 2.5e-023 Matches: 33 Glycine betaine/carnitine/choline transport ATP-binding protein OpuCA OS=Methanococcus maripaludis OX=39152 GN=opuCA PE=4 SV=1 A0A2Z5PQI8|A0A2Z5PQI8_METMI Mass: 59425 Score: 256 Expect: 9.9e-022 Matches: 32 Methyl coenzyme M reductase system component A2 OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_06700 PE=4 SV=1 A6VJS5|A6VJS5_METM7 Mass: 59377 Score: 117 Expect: 7.9e-008 Matches: 21 ABC transporter related OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_1643 PE=4 SV=1 A0A7J9PJ23|A0A7J9PJ23_METMI Mass: 59377 Score: 117 Expect: 7.9e-008 Matches: 21 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP90_001663 PE=4 SV=1 A4FYK7|A4FYK7_METM5 Mass: 59300 Score: 115 Expect: 1.2e-007 Matches: 20 ABC transporter related protein OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0985 PE=4 SV=1 A9A6A4|A9A6A4_METM6 Mass: 59413 Score: 110 Expect: 3.9e-007 Matches: 20 Methyl coenzyme M reductase system, component A2 OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=MmarC6_0271 PE=4 SV=1

2	A0A7J9NQ66|A0A7J9NQ66_METMI Mass: 59451 Score: 244 Expect: 1.6e-020 Matches: 30 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001691 PE=4 SV=1 A0A7J9PBQ6|A0A7J9PBQ6_METMI Mass: 59437 Score: 244 Expect: 1.6e-020 Matches: 30 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP91_001493 PE=4 SV=1 A0A7J9P287|A0A7J9P287_METMI Mass: 59437 Score: 244 Expect: 1.6e-020 Matches: 30 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP89_001673 PE=4 SV=1 A0A7J9PUV4|A0A7J9PUV4_METMI Mass: 59411 Score: 244 Expect: 1.6e-020 Matches: 30 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP92_001556 PE=4 SV=1
3	A6USB5|A6USB5_METVS Mass: 59530 Score: 66 Expect: 0.0092 Matches: 16 ABC transporter related OS=Methanococcus vannielii (strain ATCC 35089 / DSM 1224 / JCM 13029 / OCM 148 / SB) OX=406327 GN=Mevan_1493 PE=4 SV=1

A3. 4) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 4 from purified McrC sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Q6M0W4|Q6M0W4_METMP Mass: 56311 Score: 369 Expect: 5e-033 Matches: 35 UPF0288 protein MMP0154 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=MMP0154 PE=3 SV=1 A0A7J9S6C1|A0A7J9S6C1_METMI Mass: 56324 Score: 348 Expect: 6.3e-031 Matches: 34 UPF0288 protein HNP97_001745 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001745 PE=3 SV=1 A0A7J9NWE6|A0A7J9NWE6_METMI Mass: 56312 Score: 339 Expect: 5e-030 Matches: 33 UPF0288 protein HNP86_002165 OS=Methanococcus maripaludis OX=39152 GN=HNP86_002165 PE=3 SV=1 A0A2Z5PIK5|A0A2Z5PIK5_METMI Mass: 56312 Score: 339 Expect: 5e-030 Matches: 33 UPF0288 protein MMKA1_01590 OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_01590 PE=3 SV=1 A0A7J9P8K9|A0A7J9P8K9_METMI Mass: 56419 Score: 331 Expect: 3.1e-029 Matches: 33 UPF0288 protein HNP93_001747 OS=Methanococcus maripaludis OX=39152 GN=HNP93_001747 PE=3 SV=1 A0A7J9NLA3|A0A7J9NLA3_METMI Mass: 56296 Score: 330 Expect: 3.9e-029 Matches: 33 UPF0288 protein HNP88_000130 OS=Methanococcus maripaludis OX=39152 GN=HNP88_000130 PE=3 SV=1 A0A2Z5PUN7|A0A2Z5PUN7_METMI Mass: 56312 Score: 309 Expect: 5e-027 Matches: 30 UPF0288 protein MMOS7_01850 OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_01850 PE=3 SV=1 A0A7J9SEK5|A0A7J9SEK5_METMI Mass: 56400 Score: 285 Expect: 1.2e-024 Matches: 30 UPF0288 protein HNP96_001657 OS=Methanococcus maripaludis OX=39152 GN=HNP96_001657 PE=3 SV=1 A0A7J9PNM7|A0A7J9PNM7_METMI Mass: 56370 Score: 285 Expect: 1.2e-024 Matches: 30 UPF0288 protein HNP94_001880 OS=Methanococcus maripaludis OX=39152 GN=HNP94_001880 PE=3 SV=1 A0A2L1CAI0|A0A2L1CAI0_METMI Mass: 56354 Score: 285 Expect: 1.2e-024 Matches: 30 UPF0288 protein MMJJ_09300 OS=Methanococcus maripaludis OX=39152 GN=MMJJ_09300 PE=3 SV=1 G0H1N9|G0H1N9_METMI Mass: 56282 Score: 273 Expect: 2e-023 Matches: 28 UPF0288 protein GYY_00790 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_00790 PE=3 SV=1

	A0A7J9S5B7|A0A7J9S5B7_METMI Mass: 56440 Score: 173 Expect: 2e-013 Matches: 23 UPF0288 protein HNP89_001284 OS=Methanococcus maripaludis OX=39152 GN=HNP89_001284 PE=3 SV=1 A0A7J9PWS2|A0A7J9PWS2_METMI Mass: 56439 Score: 160 Expect: 3.9e-012 Matches: 22 UPF0288 protein HNP95_001459 OS=Methanococcus maripaludis OX=39152 GN=HNP95_001459 PE=3 SV=1 A0A7J9NIT0|A0A7J9NIT0_METMI Mass: 56430 Score: 158 Expect: 6.3e-012 Matches: 22 UPF0288 protein HNP87_001089 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001089 PE=3 SV=1 A0A7J9PC13|A0A7J9PC13_METMI Mass: 56426 Score: 158 Expect: 6.3e-012 Matches: 22 UPF0288 protein HNP91_001071 OS=Methanococcus maripaludis OX=39152 GN=HNP91_001071 PE=3 SV=1
2	Mixture 1 Total score: 302 Expect: 2.5e-026 Matches: 40 Components (only one family member shown for each component): A0A7J9SEK5|A0A7J9SEK5_METMI Mass: 56400 Score: 285 Expect: 1.2e-024 Matches: 30 UPF0288 protein HNP96_001657 OS=Methanococcus maripaludis OX=39152 GN=HNP96_001657 PE=3 SV=1 Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 61 Expect: 0.029 Matches: 11 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1
3	A4FXS0|A4FXS0_METM5 Mass: 46165 Score: 68 Expect: 0.0067 Matches: 12 Tetratricopeptide TPR_2 repeat protein OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0693 PE=4 SV=1
4	A9A8E0|A9A8E0_METM6 Mass: 56458 Score: 66 Expect: 0.011 Matches: 13 UPF0288 protein MmarC6_0796 OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=MmarC6_0796 PE=3 SV=1
5	Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 61 Expect: 0.029 Matches: 11 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1 A0A7J9NQV7|A0A7J9NQV7_METMI Mass: 61031 Score: 61 Expect: 0.029 Matches: 11 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP86_000167 PE=3 SV=1

A3. 5) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 5 from purified McrC sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Mixture 1 Total score: 139 Expect: 5e-010 Matches: 20 Components (only one family member shown for each component): G0H3A9|G0H3A9_METMI Mass: 46617 Score: 125 Expect: 1.2e-008 Matches: 12 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08625 PE=3 SV=1 Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 54 Expect: 0.14 Matches: 8 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1
2	G0H3A9|G0H3A9_METMI Mass: 46617 Score: 125 Expect: 1.2e-008 Matches: 12 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08625 PE=3 SV=1 A0A7J9NPS6|A0A7J9NPS6_METMI Mass: 46617 Score: 125 Expect: 1.2e-008 Matches: 12 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP88_001639 PE=3 SV=1 Q6LWZ9|Q6LWZ9_METMP Mass: 46617 Score: 125 Expect: 1.2e-008 Matches: 12 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrB PE=3 SV=1 A0A2L1CBB3|A0A2L1CBB3_METMI Mass: 46661 Score: 109 Expect: 5e-007 Matches: 11 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=mcB PE=3 SV=1 A0A7J9SF28|A0A7J9SF28_METMI Mass: 46631 Score: 109 Expect: 5e-007 Matches: 11 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP94_000172 PE=3 SV=1 A0A7J9NQV4|A0A7J9NQV4_METMI Mass: 46661 Score: 97 Expect: 8.4e-006 Matches: 10 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP86_000171 PE=3 SV=1 A0A7J9S0S7|A0A7J9S0S7_METMI Mass: 46661 Score: 95 Expect: 1.2e-005 Matches: 10 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP97_000800 PE=3 SV=1 A4FVX0|A4FVX0_METM5 Mass: 46563 Score: 80 Expect: 0.00039 Matches: 9 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0021 PE=3 SV=1

	A0A2Z5PK72|A0A2Z5PK72_METMI Mass: 46670 Score: 79 Expect: 0.00052 Matches: 9 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrB PE=3 SV=1 A0A7J9NHD9|A0A7J9NHD9_METMI Mass: 46695 Score: 67 Expect: 0.0075 Matches: 8 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP87_000171 PE=3 SV=1 A0A2Z5PEG7|A0A2Z5PEG7_METMI Mass: 46615 Score: 66 Expect: 0.0088 Matches: 8 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrB PE=3 SV=1
3	A9A6A9|A9A6A9_METM6 Mass: 50775 Score: 59 Expect: 0.05 Matches: 10 Probable tRNA pseudouridine synthase D OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=truD PE=3 SV=1

A3. 6) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 6 from purified McrC sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Mixture 1 Total score: 191 Expect: 3.1e-015 Matches: 30 Components (only one family member shown for each component): MMPRS224|MMP_RS02240_METMI Mass: 34878 Score: 127 Expect: 7.9e-009 Matches: 18 Auburn WO tag MM7 OS=Methanococcus maripaludis S2 A0A7J9NRP7|A0A7J9NRP7_METMI Mass: 29587 Score: 106 Expect: 9.9e-007 Matches: 13 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP86_000168 PE=3 SV=1
2	Mixture 2 Total score: 148 Expect: 6.3e-011 Matches: 27 Components (only one family member shown for each component): MMPRS224|MMP_RS02240_METMI Mass: 34878 Score: 127 Expect: 7.9e-009 Matches: 18 Auburn WO tag MM7 OS=Methanococcus maripaludis S2 A6UQK4|A6UQK4_METVS Mass: 29763 Score: 69 Expect: 0.0045 Matches: 10 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus vannielii (strain ATCC 35089 / DSM 1224 / JCM 13029 / OCM 148 / SB) OX=406327 GN=Mevan_0871 PE=3 SV=1
3	MMPRS224|MMP_RS02240_METMI Mass: 34878 Score: 127 Expect: 7.9e-009 Matches: 18 Auburn WO tag MM7 OS=Methanococcus maripaludis S2 A0A7J9NIS9|A0A7J9NIS9_METMI Mass: 34922 Score: 126 Expect: 9.9e-009 Matches: 18 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001358 PE=4 SV=1 A0A2L1C9H0|A0A2L1C9H0_METMI Mass: 34949 Score: 126 Expect: 9.9e-009 Matches: 18 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=HNP86_001650 PE=4 SV=1 Q6M050|Q6M050_METMP Mass: 34949 Score: 126 Expect: 9.9e-009 Matches: 18 Uncharacterized protein OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=MMP0421 PE=4 SV=1 MMPRSt224|MMP_RS02240_METMI Mass: 40900 Score: 121 Expect: 3.1e-008 Matches: 18 Auburn Tagged MM7 OS=Methanococcus maripaludis S2 A0A7J9S333|A0A7J9S333_METMI Mass: 34936 Score: 111 Expect: 3.1e-007 Matches: 17 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001461 PE=4 SV=1

	A0A7J9PN18|A0A7J9PN18_METMI Mass: 34814 Score: 108 Expect: 6.3e-007 Matches: 17 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP94_001513 PE=4 SV=1 G0H350|G0H350_METMI Mass: 34921 Score: 102 Expect: 2.5e-006 Matches: 16 Methanogenesis marker protein 7 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_02180 PE=4 SV=1 A0A2Z5PFC4|A0A2Z5PFC4_METMI Mass: 34921 Score: 102 Expect: 2.5e-006 Matches: 16 Uncharacterized protein OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_04360 PE=4 SV=1 A0A2Z5PQA8|A0A2Z5PQA8_METMI Mass: 34921 Score: 102 Expect: 2.5e-006 Matches: 16 Uncharacterized protein OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_04400 PE=4 SV=1 A0A7J9S9F8|A0A7J9S9F8_METMI Mass: 34939 Score: 94 Expect: 1.7e-005 Matches: 15 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP92_000953 PE=4 SV=1
4	A0A7J9NRP7|A0A7J9NRP7_METMI Mass: 29587 Score: 106 Expect: 9.9e-007 Matches: 13 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP86_000168 PE=3 SV=1 Q6LWZ6|Q6LWZ6_METMP Mass: 29587 Score: 106 Expect: 9.9e-007 Matches: 13 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrG PE=3 SV=1 A0A2L1CBG2|A0A2L1CBG2_METMI Mass: 29605 Score: 92 Expect: 2.5e-005 Matches: 12 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=mcrG PE=3 SV=1 A0A7J9RZ90|A0A7J9RZ90_METMI Mass: 29619 Score: 92 Expect: 2.5e-005 Matches: 12 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP93_000169 PE=3 SV=1 A0A7J9S447|A0A7J9S447_METMI Mass: 29619 Score: 92 Expect: 2.5e-005 Matches: 12 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP87_000168 PE=3 SV=1 G0H3B2|G0H3B2_METMI Mass: 29718 Score: 92 Expect: 2.6e-005 Matches: 12 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08640 PE=3 SV=1 A0A2Z5PJC7|A0A2Z5PJC7_METMI Mass: 29633 Score: 90 Expect: 3.9e-005 Matches: 12 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrG PE=3 SV=1 A0A2Z5PN58|A0A2Z5PN58_METMI Mass: 29649 Score: 81 Expect: 0.00032 Matches: 11 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrG PE=3 SV=1

	A0A2L1CBG2m|A0A2L1CBG2m_METMI Mass: 33971 Score: 78 Expect: 0.00061 Matches: 11 modified Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=mcrG PE=4 SV=1 A0A7J9NYB3|A0A7J9NYB3_METMI Mass: 29637 Score: 77 Expect: 0.00077 Matches: 11 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP89_000171 PE=3 SV=1
5	A6UQK4|A6UQK4_METVS Mass: 29763 Score: 69 Expect: 0.0045 Matches: 10 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus vannielii (strain ATCC 35089 / DSM 1224 / JCM 13029 / OCM 148 / SB) OX=406327 GN=Mevan_0871 PE=3 SV=1 P07963|MCRG_METVA Mass: 29763 Score: 69 Expect: 0.0045 Matches: 10 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus vannielii OX=2187 GN=mcrG PE=3 SV=1
6	A0A7J9PHI7|A0A7J9PHI7_METMI Mass: 34922 Score: 58 Expect: 0.056 Matches: 11 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP90_001125 PE=4 SV=1 A6VJ56|A6VJ56_METM7 Mass: 34922 Score: 58 Expect: 0.056 Matches: 11 Uncharacterized protein OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_1419 PE=4 SV=1

A3. 7) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 7 from purified McrC sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	A0A7J9NRP7|A0A7J9NRP7_METMI Mass: 29587 Score: 240 Expect: 3.9e-020 Matches: 24 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP86_000168 PE=3 SV=1 Q6LWZ6|Q6LWZ6_METMP Mass: 29587 Score: 240 Expect: 3.9e-020 Matches: 24 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrG PE=3 SV=1 A0A7J9S447|A0A7J9S447_METMI Mass: 29619 Score: 200 Expect: 3.9e-016 Matches: 21 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP87_000168 PE=3 SV=1 A0A7J9NYB3|A0A7J9NYB3_METMI Mass: 29637 Score: 180 Expect: 3.9e-014 Matches: 20 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP89_000171 PE=3 SV=1 A6VHE6|A6VHE6_METM7 Mass: 29619 Score: 137 Expect: 7.9e-010 Matches: 17 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_0805 PE=3 SV=1 A0A7J9PFI7|A0A7J9PFI7_METMI Mass: 29619 Score: 137 Expect: 7.9e-010 Matches: 17 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP90_000388 PE=3 SV=1 A6UQK4|A6UQK4_METVS Mass: 29763 Score: 131 Expect: 3.1e-009 Matches: 17 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus vannielii (strain ATCC 35089 / DSM 1224 / JCM 13029 / OCM 148 / SB) OX=406327 GN=Mevan_0871 PE=3 SV=1 P07963|MCRG_METVA Mass: 29763 Score: 131 Expect: 3.1e-009 Matches: 17 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus vannielii OX=2187 GN=mcrG PE=3 SV=1
2	. A0A2L1CBG2|A0A2L1CBG2_METMI Mass: 29605 Score: 234 Expect: 1.6e-019 Matches: 23 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=mcrG PE=3 SV=1 A0A7J9RZ90|A0A7J9RZ90_METMI Mass: 29619 Score: 234 Expect: 1.6e-019 Matches: 23 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP93_000169 PE=3 SV=1 G0H3B2|G0H3B2_METMI Mass: 29718 Score: 234 Expect: 1.6e-019 Matches: 23 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08640 PE=3 SV=1

	A0A2Z5PJC7|A0A2Z5PJC7_METMI Mass: 29633 Score: 231 Expect: 3.1e-019 Matches: 23 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrG PE=3 SV=1 A0A2L1CBG2m|A0A2L1CBG2m_METMI Mass: 33971 Score: 214 Expect: 1.6e-017 Matches: 22 modified Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=mcrG PE=4 SV=1 A0A2Z5PN58|A0A2Z5PN58_METMI Mass: 29649 Score: 201 Expect: 3.1e-016 Matches: 21 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrG PE=3 SV=1
3	A4FVW7|A4FVW7_METM5 Mass: 29703 Score: 137 Expect: 7.9e-010 Matches: 17 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0018 PE=3 SV=1 A9A9A3|A9A9A3_METM6 Mass: 29704 Score: 105 Expect: 1.2e-006 Matches: 14 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=MmarC6_1112 PE=3 SV=1

A3. 8) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 8 from purified McrC sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Q6LWZ7|Q6LWZ7_METMP Mass: 21340 Score: 121 Expect: 3.1e-008 Matches: 13 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrC PE=4 SV=1 G0H3B1|G0H3B1_METMI Mass: 21340 Score: 121 Expect: 3.1e-008 Matches: 13 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08635 PE=4 SV=1 A0A2Z5PM12|A0A2Z5PM12_METMI Mass: 21310 Score: 121 Expect: 3.1e-008 Matches: 13 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrC PE=4 SV=1 A0A7J9NGE5|A0A7J9NGE5_METMI Mass: 21319 Score: 121 Expect: 3.1e-008 Matches: 13 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=HNP87_000169 PE=4 SV=1 A0A2L1CBQ8|A0A2L1CBQ8_METMI Mass: 21340 Score: 121 Expect: 3.1e-008 Matches: 13 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=mcrC PE=4 SV=1 A0A2Z5PNK7|A0A2Z5PNK7_METMI Mass: 21340 Score: 121 Expect: 3.1e-008 Matches: 13 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrC PE=4 SV=1 A4FVW8|A4FVW8_METM5 Mass: 21428 Score: 107 Expect: 7.9e-007 Matches: 12 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0019 PE=4 SV=1 A0A7J9P9K7|A0A7J9P9K7_METMI Mass: 21292 Score: 93 Expect: 1.9e-005 Matches: 11 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=HNP89_000172 PE=4 SV=1 A6UQK3|A6UQK3_METVS Mass: 21354 Score: 81 Expect: 0.00032 Matches: 10 Methyl-coenzyme M reductase operon protein C OS=Methanococcus vannielii (strain ATCC 35089 / DSM 1224 / JCM 13029 / OCM 148 / SB) OX=406327 GN=Mevan_0870 PE=4 SV=1 P07960|MCRC_METVA Mass: 21354 Score: 81 Expect: 0.00032 Matches: 10 Methyl-coenzyme M reductase operon protein C OS=Methanococcus vannielii OX=2187 GN=mcrC PE=4 SV=1 A9A9A4|A9A9A4_METM6 Mass: 21368 Score: 70 Expect: 0.0043 Matches: 9 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=MmarC6_1113 PE=4 SV=1

2	A0A7J9PFQ9|A0A7J9PFQ9_METMI Mass: 21305 Score: 94 Expect: 1.4e-005 Matches: 11 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=HNP90_000387 PE=4 SV=1 A6VHE5|A6VHE5_METM7 Mass: 21305 Score: 94 Expect: 1.4e-005 Matches: 11 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_0804 PE=4 SV=1

A3. 9) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 9 from purified McrC sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Q6LWZ7|Q6LWZ7_METMP Mass: 21340 Score: 90 Expect: 3.7e-005 Matches: 8 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrC PE=4 SV=1 A4FVW8|A4FVW8_METM5 Mass: 21428 Score: 90 Expect: 3.7e-005 Matches: 8 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0019 PE=4 SV=1 G0H3B1|G0H3B1_METMI Mass: 21340 Score: 90 Expect: 3.7e-005 Matches: 8 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08635 PE=4 SV=1 A0A2Z5PM12|A0A2Z5PM12_METMI Mass: 21310 Score: 90 Expect: 3.7e-005 Matches: 8 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrC PE=4 SV=1 A0A7J9NGE5|A0A7J9NGE5_METMI Mass: 21319 Score: 90 Expect: 3.7e-005 Matches: 8 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=HNP87_000169 PE=4 SV=1 A0A2L1CBQ8|A0A2L1CBQ8_METMI Mass: 21340 Score: 90 Expect: 3.7e-005 Matches: 8 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=mcrC PE=4 SV=1 A0A2Z5PNK7|A0A2Z5PNK7_METMI Mass: 21340 Score: 90 Expect: 3.7e-005 Matches: 8

A3. 10) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 10 from purified McrC sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	. A0A7J9S5Z5|A0A7J9S5Z5_METMI Mass: 21046 Score: 98 Expect: 5.7e-006 Matches: 10 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP92_001514 PE=4 SV=1 A0A7J9P1C1|A0A7J9P1C1_METMI Mass: 21064 Score: 97 Expect: 7.7e-006 Matches: 10 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP89_001714 PE=4 SV=1 A0A2Z5PMC3|A0A2Z5PMC3_METMI Mass: 21063 Score: 97 Expect: 8.4e-006 Matches: 10 Uncharacterized protein OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_07070 PE=4 SV=1 A0A7J9NM23|A0A7J9NM23_METMI Mass: 21049 Score: 97 Expect: 8.4e-006 Matches: 10 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP88_000689 PE=4 SV=1 A0A7J9PBM9|A0A7J9PBM9_METMI Mass: 21078 Score: 97 Expect: 8.4e-006 Matches: 10 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP91_001453 PE=4 SV=1 G0H411|G0H411_METMI Mass: 21063 Score: 97 Expect: 8.4e-006 Matches: 10 Methanogenesis marker protein 17 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_02990 PE=4 SV=1 A0A7J9NQB0|A0A7J9NQB0_METMI Mass: 21064 Score: 97 Expect: 8.4e-006 Matches: 10 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001731 PE=4 SV=1 A0A2Z5PJQ8|A0A2Z5PJQ8_METMI Mass: 21063 Score: 97 Expect: 8.4e-006 Matches: 10 Uncharacterized protein OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_07020 PE=4 SV=1 A0A7J9S1E9|A0A7J9S1E9_METMI Mass: 21063 Score: 97 Expect: 8.4e-006 Matches: 10 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001264 PE=4 SV=1 A0A2L1C8U1|A0A2L1C8U1_METMI Mass: 21077 Score: 97 Expect: 8.4e-006 Matches: 10 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP94_001155 PE=4 SV=1 Q6LZH2|Q6LZH2_METMP Mass: 21063 Score: 97 Expect: 8.4e-006 Matches: 10 Uncharacterized protein OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=MMP0656 PE=4 SV=1

	A0A7J9PUS5|A0A7J9PUS5_METMI Mass: 21037 Score: 96 Expect: 9.5e-006 Matches: 10 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP95_001745 PE=4 SV=1 A9A7C8|A9A7C8_METM6 Mass: 21078 Score: 95 Expect: 1.3e-005 Matches: 10 Uncharacterized protein OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=MmarC6_0231 PE=4 SV=1 A0A7J9NUG4|A0A7J9NUG4_METMI Mass: 21062 Score: 80 Expect: 0.00039 Matches: 9 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP86_001066 PE=4 SV=1 A0A7J9P6S6|A0A7J9P6S6_METMI Mass: 21034 Score: 80 Expect: 0.00039 Matches: 9 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP93_001139 PE=4 SV=1 A6VJW2|A6VJW2_METM7 Mass: 21106 Score: 63 Expect: 0.019 Matches: 8 Uncharacterized protein OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_1682 PE=4 SV=1 A0A7J9PHM4|A0A7J9PHM4_METMI Mass: 21106 Score: 63 Expect: 0.019 Matches: 8 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP90_001622 PE=4 SV=1

A3. 11) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 1 from peak one of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Mixture 1 Total score: 122 Expect: 2.5e-008 Matches: 23 Components (only one family member shown for each component): Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 108 Expect: 6.3e-007 Matches: 13 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1 Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 50 Expect: 0.43 Matches: 10 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1
2	Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 108 Expect: 6.3e-007 Matches: 13 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1 A0A7J9NQV7|A0A7J9NQV7_METMI Mass: 61031 Score: 108 Expect: 6.3e-007 Matches: 13 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP86_000167 PE=3 SV=1 G0H3B3|G0H3B3_METMI Mass: 61040 Score: 96 Expect: 1e-005 Matches: 12 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08645 PE=3 SV=1 A0A2L1CBB0|A0A2L1CBB0_METMI Mass: 61038 Score: 96 Expect: 1e-005 Matches: 12 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=mcrA PE=3 SV=1 A0A2Z5PTJ7|A0A2Z5PTJ7_METMI Mass: 61024 Score: 96 Expect: 1e-005 Matches: 12 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrA PE=3 SV=1 A0A7J9PKB0|A0A7J9PKB0_METMI Mass: 61040 Score: 96 Expect: 1e-005 Matches: 12 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP93_000168 PE=3 SV=1 A0A7J9S9S4|A0A7J9S9S4_METMI Mass: 61024 Score: 96 Expect: 1e-005 Matches: 12 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP96_000849 PE=3 SV=1 A0A7J9S5U7|A0A7J9S5U7_METMI Mass: 61031 Score: 94 Expect: 1.4e-005 Matches: 12 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP92_000701 PE=3 SV=1

	A0A7J9NYX4|A0A7J9NYX4_METMI Mass: 60983 Score: 94 Expect: 1.4e-005 Matches: 12 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP87_000167 PE=3 SV=1 A0A2Z5PKS6|A0A2Z5PKS6_METMI Mass: 61016 Score: 84 Expect: 0.00014 Matches: 11 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrA PE=3 SV=1 H7CHX3|H7CHX3_METMI Mass: 41475 Score: 61 Expect: 0.034 Matches: 8 Coenzyme-B sulfoethylthiotransferase (Fragment) OS=Methanococcus maripaludis OX=39152 GN=mcrA PE=3 SV=1
3	A4FVW6|A4FVW6_METM5 Mass: 61055 Score: 72 Expect: 0.0028 Matches: 10 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0017 PE=3 SV=1
4	A6VHE7|A6VHE7_METM7 Mass: 61100 Score: 60 Expect: 0.037 Matches: 9 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_0806 PE=3 SV=1 A0A7J9PJ28|A0A7J9PJ28_METMI Mass: 61100 Score: 60 Expect: 0.037 Matches: 9 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP90_000389 PE=3 SV=1

A3. 12) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 2 from peak one of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	G0H3A9|G0H3A9_METMI Mass: 46617 Score: 138 Expect: 6.3e-010 Matches: 13 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08625 PE=3 SV=1 A0A7J9NPS6|A0A7J9NPS6_METMI Mass: 46617 Score: 138 Expect: 6.3e-010 Matches: 13 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP88_001639 PE=3 SV=1 Q6LWZ9|Q6LWZ9_METMP Mass: 46617 Score: 138 Expect: 6.3e-010 Matches: 13 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrB PE=3 SV=1 A0A2L1CBB3|A0A2L1CBB3_METMI Mass: 46661 Score: 123 Expect: 2e-008 Matches: 12 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=mcB PE=3 SV=1 A0A7J9SF28|A0A7J9SF28_METMI Mass: 46631 Score: 123 Expect: 2e-008 Matches: 12 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP94_000172 PE=3 SV=1 A0A7J9NQV4|A0A7J9NQV4_METMI Mass: 46661 Score: 109 Expect: 5e-007 Matches: 11 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP86_000171 PE=3 SV=1 A0A7J9S0S7|A0A7J9S0S7_METMI Mass: 46661 Score: 94 Expect: 1.4e-005 Matches: 10 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP97_000800 PE=3 SV=1 A0A2Z5PK72|A0A2Z5PK72_METMI Mass: 46670 Score: 92 Expect: 2.5e-005 Matches: 10 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrB PE=3 SV=1 A0A7J9NHD9|A0A7J9NHD9_METMI Mass: 46695 Score: 80 Expect: 0.00038 Matches: 9 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP87_000171 PE=3 SV=1 A0A2Z5PEG7|A0A2Z5PEG7_METMI Mass: 46615 Score: 79 Expect: 0.0005 Matches: 9 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrB PE=3 SV=1

	A9A9A6|A9A9A6_METM6 Mass: 46568 Score: 66 Expect: 0.009 Matches: 8 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=MmarC6_1115 PE=3 SV=1

A3. 13) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 3 from peak one of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	A0A7J9PHN1|A0A7J9PHN1_METMI Mass: 21488 Score: 60 Expect: 0.036 Matches: 6 Heterodisulfide reductase subunit C OS=Methanococcus maripaludis OX=39152 GN=HNP90_001059 PE=3 SV=1 A6VG95|A6VG95_METM7 Mass: 21488 Score: 60 Expect: 0.036 Matches: 6 CoB--CoM heterodisulfide reductase OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_0402 PE=3 SV=1

A3. 14) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 1 from peak two of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Mixture 1 Total score: 247 Expect: 7.9e-021 Matches: 40 Components (only one family member shown for each component): Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 137 Expect: 7.9e-010 Matches: 22 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1 Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 128 Expect: 6.3e-009 Matches: 18 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1
2	Mixture 2 Total score: 197 Expect: 7.9e-016 Matches: 37 Components (only one family member shown for each component): Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 137 Expect: 7.9e-010 Matches: 22 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1 A4FVW6|A4FVW6_METM5 Mass: 61055 Score: 86 Expect: 9.5e-005 Matches: 15 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0017 PE=3 SV=1
3	Mixture 3 Total score: 173 Expect: 2e-013 Matches: 35 Components (only one family member shown for each component): Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 137 Expect: 7.9e-010 Matches: 22 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1 A6VHE7|A6VHE7_METM7 Mass: 61100 Score: 67 Expect: 0.0075 Matches: 13 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_0806 PE=3 SV=1
4	Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 137 Expect: 7.9e-010 Matches: 22 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1 A0A2Z5PV80|A0A2Z5PV80_METMI Mass: 59453 Score: 137 Expect: 7.9e-010 Matches: 22 Methyl coenzyme M reductase system component A2 OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_06650 PE=4 SV=1

	G0H4C2|G0H4C2_METMI Mass: 59453 Score: 137 Expect: 7.9e-010 Matches: 22 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_03370 PE=4 SV=1 A0A7J9NUM7|A0A7J9NUM7_METMI Mass: 59440 Score: 137 Expect: 7.9e-010 Matches: 22 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP86_001138 PE=4 SV=1 A0A7J9RZV0|A0A7J9RZV0_METMI Mass: 59426 Score: 137 Expect: 7.9e-010 Matches: 22 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001226 PE=4 SV=1 A0A2L1C9A1|A0A2L1C9A1_METMI Mass: 59453 Score: 137 Expect: 7.9e-010 Matches: 22 Glycine betaine/carnitine/choline transport ATP-binding protein OpuCA OS=Methanococcus maripaludis OX=39152 GN=opuCA PE=4 SV=1 A0A2Z5PQI8|A0A2Z5PQI8_METMI Mass: 59425 Score: 125 Expect: 1.2e-008 Matches: 21 Methyl coenzyme M reductase system component A2 OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_06700 PE=4 SV=1 A0A7J9NQ66|A0A7J9NQ66_METMI Mass: 59451 Score: 103 Expect: 2e-006 Matches: 18 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001691 PE=4 SV=1 A0A7J9PBQ6|A0A7J9PBQ6_METMI Mass: 59437 Score: 103 Expect: 2e-006 Matches: 18 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP91_001493 PE=4 SV=1 A0A7J9P287|A0A7J9P287_METMI Mass: 59437 Score: 103 Expect: 2e-006 Matches: 18 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP89_001673 PE=4 SV=1 A0A7J9PUV4|A0A7J9PUV4_METMI Mass: 59411 Score: 103 Expect: 2e-006 Matches: 18 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP92_001556 PE=4 SV=1
5	Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 128 Expect: 6.3e-009 Matches: 18 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1 A0A7J9NQV7|A0A7J9NQV7_METMI Mass: 61031 Score: 128 Expect: 6.3e-009 Matches: 18 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP86_000167 PE=3 SV=1 G0H3B3|G0H3B3_METMI Mass: 61040 Score: 117 Expect: 7.9e-008 Matches: 17 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08645 PE=3 SV=1 A0A2L1CBB0|A0A2L1CBB0_METMI Mass: 61038 Score: 117 Expect: 7.9e-008 Matches: 17 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=mcrA PE=3 SV=1

	A0A2Z5PTJ7|A0A2Z5PTJ7_METMI Mass: 61024 Score: 117 Expect: 7.9e-008 Matches: 17 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrA PE=3 SV=1 A0A7J9PKB0|A0A7J9PKB0_METMI Mass: 61040 Score: 117 Expect: 7.9e-008 Matches: 17 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP93_000168 PE=3 SV=1 A0A7J9S9S4|A0A7J9S9S4_METMI Mass: 61024 Score: 117 Expect: 7.9e-008 Matches: 17 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP96_000849 PE=3 SV=1 A0A2Z5PKS6|A0A2Z5PKS6_METMI Mass: 61016 Score: 117 Expect: 7.9e-008 Matches: 17 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrA PE=3 SV=1 A0A7J9NYX4|A0A7J9NYX4_METMI Mass: 60983 Score: 104 Expect: 1.6e-006 Matches: 16 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP87_000167 PE=3 SV=1 A0A7J9S5U7|A0A7J9S5U7_METMI Mass: 61031 Score: 95 Expect: 1.3e-005 Matches: 15 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP92_000701 PE=3 SV=1
6	A4FVW6|A4FVW6_METM5 Mass: 61055 Score: 86 Expect: 9.5e-005 Matches: 15 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0017 PE=3 SV=1
7	A6VHE7|A6VHE7_METM7 Mass: 61100 Score: 67 Expect: 0.0075 Matches: 13 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_0806 PE=3 SV=1 A0A7J9PJ28|A0A7J9PJ28_METMI Mass: 61100 Score: 67 Expect: 0.0075 Matches: 13 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP90_000389 PE=3 SV=1
8	A4FYK7|A4FYK7_METM5 Mass: 59300 Score: 66 Expect: 0.009 Matches: 14 ABC transporter related protein OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0985 PE=4 SV=1

A3. 15) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 2 from peak two of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Q6M0W4|Q6M0W4_METMP Mass: 56311 Score: 202 Expect: 2.5e-016 Matches: 22 UPF0288 protein MMP0154 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=MMP0154 PE=3 SV=1 A0A7J9S6C1|A0A7J9S6C1_METMI Mass: 56324 Score: 185 Expect: 1.2e-014 Matches: 21 UPF0288 protein HNP97_001745 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001745 PE=3 SV=1 A0A7J9NWE6|A0A7J9NWE6_METMI Mass: 56312 Score: 180 Expect: 3.9e-014 Matches: 20 UPF0288 protein HNP86_002165 OS=Methanococcus maripaludis OX=39152 GN=HNP86_002165 PE=3 SV=1 A0A2Z5PIK5|A0A2Z5PIK5_METMI Mass: 56312 Score: 180 Expect: 3.9e-014 Matches: 20 UPF0288 protein MMKA1_01590 OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_01590 PE=3 SV=1 A0A7J9P8K9|A0A7J9P8K9_METMI Mass: 56419 Score: 173 Expect: 2e-013 Matches: 20 UPF0288 protein HNP93_001747 OS=Methanococcus maripaludis OX=39152 GN=HNP93_001747 PE=3 SV=1 A0A7J9NLA3|A0A7J9NLA3_METMI Mass: 56296 Score: 171 Expect: 3.1e-013 Matches: 20 UPF0288 protein HNP88_000130 OS=Methanococcus maripaludis OX=39152 GN=HNP88_000130 PE=3 SV=1 A0A2Z5PUN7|A0A2Z5PUN7_METMI Mass: 56312 Score: 168 Expect: 6.3e-013 Matches: 19 UPF0288 protein MMOS7_01850 OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_01850 PE=3 SV=1 A0A7J9SEK5|A0A7J9SEK5_METMI Mass: 56400 Score: 150 Expect: 3.9e-011 Matches: 18 UPF0288 protein HNP96_001657 OS=Methanococcus maripaludis OX=39152 GN=HNP96_001657 PE=3 SV=1 A0A7J9PNM7|A0A7J9PNM7_METMI Mass: 56370 Score: 150 Expect: 3.9e-011 Matches: 18 UPF0288 protein HNP94_001880 OS=Methanococcus maripaludis OX=39152 GN=HNP94_001880 PE=3 SV=1 A0A2L1CAI0|A0A2L1CAI0_METMI Mass: 56354 Score: 150 Expect: 3.9e-011 Matches: 18 UPF0288 protein MMJJ_09300 OS=Methanococcus maripaludis OX=39152 GN=MMJJ_09300 PE=3 SV=1 G0H1N9|G0H1N9_METMI Mass: 56282 Score: 140 Expect: 3.9e-010 Matches: 17 UPF0288 protein GYY_00790 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_00790 PE=3 SV=1

	A0A7J9PWS2|A0A7J9PWS2_METMI Mass: 56439 Score: 76 Expect: 0.0009 Matches: 12 UPF0288 protein HNP95_001459 OS=Methanococcus maripaludis OX=39152 GN=HNP95_001459 PE=3 SV=1 A0A7J9S5B7|A0A7J9S5B7_METMI Mass: 56440 Score: 76 Expect: 0.0009 Matches: 12 UPF0288 protein HNP89_001284 OS=Methanococcus maripaludis OX=39152 GN=HNP89_001284 PE=3 SV=1 A0A7J9NIT0|A0A7J9NIT0_METMI Mass: 56430 Score: 65 Expect: 0.011 Matches: 11 UPF0288 protein HNP87_001089 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001089 PE=3 SV=1 A0A7J9PC13|A0A7J9PC13_METMI Mass: 56426 Score: 65 Expect: 0.012 Matches: 11 UPF0288 protein HNP91_001071 OS=Methanococcus maripaludis OX=39152 GN=HNP91_001071 PE=3 SV=1

A3. 16) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 3 from peak two of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	G0H3A9|G0H3A9_METMI Mass: 46617 Score: 88 Expect: 5.6e-005 Matches: 10 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08625 PE=3 SV=1 A0A2L1CBB3|A0A2L1CBB3_METMI Mass: 46661 Score: 88 Expect: 5.6e-005 Matches: 10 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=mcB PE=3 SV=1 A0A7J9SF28|A0A7J9SF28_METMI Mass: 46631 Score: 88 Expect: 5.6e-005 Matches: 10 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP94_000172 PE=3 SV=1 A0A7J9NPS6|A0A7J9NPS6_METMI Mass: 46617 Score: 88 Expect: 5.6e-005 Matches: 10 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP88_001639 PE=3 SV=1 Q6LWZ9|Q6LWZ9_METMP Mass: 46617 Score: 88 Expect: 5.6e-005 Matches: 10 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrB PE=3 SV=1 A0A7J9S0S7|A0A7J9S0S7_METMI Mass: 46661 Score: 76 Expect: 0.00097 Matches: 9 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP97_000800 PE=3 SV=1 A0A7J9NQV4|A0A7J9NQV4_METMI Mass: 46661 Score: 66 Expect: 0.011 Matches: 8 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP86_000171 PE=3 SV=1 A4FVX0|A4FVX0_METM5 Mass: 46563 Score: 63 Expect: 0.02 Matches: 8 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0021 PE=3 SV=1 A0A2Z5PK72|A0A2Z5PK72_METMI Mass: 46670 Score: 62 Expect: 0.027 Matches: 8 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrB PE=3 SV=1

A3. 17) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no:4 from peak two of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	MMPRS224|MMP_RS02240_METMI Mass: 34878 Score: 91 Expect: 3.1e-005 Matches: 7 Auburn WO tag MM7 OS=Methanococcus maripaludis S2 A0A7J9NIS9|A0A7J9NIS9_METMI Mass: 34922 Score: 91 Expect: 3.2e-005 Matches: 7 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001358 PE=4 SV=1 A0A2L1C9H0|A0A2L1C9H0_METMI Mass: 34949 Score: 91 Expect: 3.2e-005 Matches: 7 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=HNP86_001650 PE=4 SV=1 Q6M050|Q6M050_METMP Mass: 34949 Score: 91 Expect: 3.2e-005 Matches: 7 Uncharacterized protein OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=MMP0421 PE=4 SV=1 A0A7J9S9F8|A0A7J9S9F8_METMI Mass: 34939 Score: 90 Expect: 3.9e-005 Matches: 7 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP92_000953 PE=4 SV=1 A0A2Z5PFC4|A0A2Z5PFC4_METMI Mass: 34921 Score: 73 Expect: 0.0021 Matches: 6 Uncharacterized protein OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_04360 PE=4 SV=1 A0A2Z5PQA8|A0A2Z5PQA8_METMI Mass: 34921 Score: 73 Expect: 0.0021 Matches: 6 Uncharacterized protein OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_04400 PE=4 SV=1 A0A7J9S333|A0A7J9S333_METMI Mass: 34936 Score: 68 Expect: 0.0065 Matches: 6 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001461 PE=4 SV=1 A0A7J9PHI7|A0A7J9PHI7_METMI Mass: 34922 Score: 64 Expect: 0.017 Matches: 6 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP90_001125 PE=4 SV=1 A6VJ56|A6VJ56_METM7 Mass: 34922 Score: 64 Expect: 0.017 Matches: 6 Uncharacterized protein OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_1419 PE=4 SV=1 A0A7J9PN18|A0A7J9PN18_METMI Mass: 34814 Score: 63 Expect: 0.019 Matches: 6 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP94_001513 PE=4 SV=1

A3. 18) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no:5 from peak two of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	A0A7J9NRP7|A0A7J9NRP7_METMI Mass: 29587 Score: 256 Expect: 9.9e-022 Matches: 20 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP86_000168 PE=3 SV=1 Q6LWZ6|Q6LWZ6_METMP Mass: 29587 Score: 256 Expect: 9.9e-022 Matches: 20 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrG PE=3 SV=1 A0A2L1CBG2|A0A2L1CBG2_METMI Mass: 29605 Score: 233 Expect: 2e-019 Matches: 19 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=mcrG PE=3 SV=1 A0A7J9RZ90|A0A7J9RZ90_METMI Mass: 29619 Score: 233 Expect: 2e-019 Matches: 19 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP93_000169 PE=3 SV=1 G0H3B2|G0H3B2_METMI Mass: 29718 Score: 233 Expect: 2e-019 Matches: 19 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08640 PE=3 SV=1 A0A2Z5PJC7|A0A2Z5PJC7_METMI Mass: 29633 Score: 230 Expect: 3.9e-019 Matches: 19 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrG PE=3 SV=1 A0A2L1CBG2m|A0A2L1CBG2m_METMI Mass: 33971 Score: 213 Expect: 2e-017 Matches: 18 modified Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=mcrG PE=4 SV=1 A0A2Z5PN58|A0A2Z5PN58_METMI Mass: 29649 Score: 198 Expect: 6.3e-016 Matches: 17 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrG PE=3 SV=1 A0A7J9S447|A0A7J9S447_METMI Mass: 29619 Score: 198 Expect: 6.3e-016 Matches: 17 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP87_000168 PE=3 SV=1 A0A7J9NYB3|A0A7J9NYB3_METMI Mass: 29637 Score: 177 Expect: 7.9e-014 Matches: 16 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP89_000171 PE=3 SV=1 A6VHE6|A6VHE6_METM7 Mass: 29619 Score: 164 Expect: 1.6e-012 Matches: 15 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_0805 PE=3 SV=1

	A0A7J9PFI7|A0A7J9PFI7_METMI Mass: 29619 Score: 164 Expect: 1.6e-012 Matches: 15 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP90_000388 PE=3 SV=1 A4FVW7|A4FVW7_METM5 Mass: 29703 Score: 149 Expect: 5e-011 Matches: 14 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0018 PE=3 SV=1 A6UQK4|A6UQK4_METVS Mass: 29763 Score: 143 Expect: 2e-010 Matches: 14 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus vannielii (strain ATCC 35089 / DSM 1224 / JCM 13029 / OCM 148 / SB) OX=406327 GN=Mevan_0871 PE=3 SV=1 P07963|MCRG_METVA Mass: 29763 Score: 143 Expect: 2e-010 Matches: 14 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus vannielii OX=2187 GN=mcrG PE=3 SV=1 A9A9A3|A9A9A3_METM6 Mass: 29704 Score: 116 Expect: 9.9e-008 Matches: 12 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=MmarC6_1112 PE=3 SV=1

A3. 19) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no:6 from peak two of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Q6LWZ7|Q6LWZ7_METMP Mass: 21340 Score: 105 Expect: 1.2e-006 Matches: 8 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrC PE=4 SV=1 G0H3B1|G0H3B1_METMI Mass: 21340 Score: 105 Expect: 1.2e-006 Matches: 8 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08635 PE=4 SV=1 A0A2Z5PM12|A0A2Z5PM12_METMI Mass: 21310 Score: 105 Expect: 1.2e-006 Matches: 8 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrC PE=4 SV=1 A0A2L1CBQ8|A0A2L1CBQ8_METMI Mass: 21340 Score: 105 Expect: 1.2e-006 Matches: 8 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=mcrC PE=4 SV=1 A0A2Z5PNK7|A0A2Z5PNK7_METMI Mass: 21340 Score: 105 Expect: 1.2e-006 Matches: 8 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrC PE=4 SV=1 A0A7J9NGE5|A0A7J9NGE5_METMI Mass: 21319 Score: 75 Expect: 0.0014 Matches: 7 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=HNP87_000169 PE=4 SV=1 A4FVW8|A4FVW8_METM5 Mass: 21428 Score: 60 Expect: 0.044 Matches: 6 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0019 PE=4 SV=1 A0A7J9P9K7|A0A7J9P9K7_METMI Mass: 21292 Score: 59 Expect: 0.046 Matches: 6 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=HNP89_000172 PE=4 SV=1

A3. 20) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no:7 from peak two of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	A0A7J9NQB0|A0A7J9NQB0_METMI Mass: 21064 Score: 84 Expect: 0.00015 Matches: 9 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001731 PE=4 SV=1 A0A7J9PUS5|A0A7J9PUS5_METMI Mass: 21037 Score: 84 Expect: 0.00017 Matches: 9 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP95_001745 PE=4 SV=1 A9A7C8|A9A7C8_METM6 Mass: 21078 Score: 82 Expect: 0.00022 Matches: 9 Uncharacterized protein OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=MmarC6_0231 PE=4 SV=1 A0A7J9S5Z5|A0A7J9S5Z5_METMI Mass: 21046 Score: 72 Expect: 0.0025 Matches: 8 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP92_001514 PE=4 SV=1 A0A7J9P1C1|A0A7J9P1C1_METMI Mass: 21064 Score: 71 Expect: 0.0032 Matches: 8 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP89_001714 PE=4 SV=1 A0A2Z5PMC3|A0A2Z5PMC3_METMI Mass: 21063 Score: 70 Expect: 0.0036 Matches: 8 Uncharacterized protein OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_07070 PE=4 SV=1 A0A7J9NM23|A0A7J9NM23_METMI Mass: 21049 Score: 70 Expect: 0.0036 Matches: 8 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP88_000689 PE=4 SV=1 A0A7J9PBM9|A0A7J9PBM9_METMI Mass: 21078 Score: 70 Expect: 0.0036 Matches: 8 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP91_001453 PE=4 SV=1 G0H411|G0H411_METMI Mass: 21063 Score: 70 Expect: 0.0036 Matches: 8 Methanogenesis marker protein 17 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_02990 PE=4 SV=1 A0A2Z5PJQ8|A0A2Z5PJQ8_METMI Mass: 21063 Score: 70 Expect: 0.0036 Matches: 8 Uncharacterized protein OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_07020 PE=4 SV=1 A0A7J9S1E9|A0A7J9S1E9_METMI Mass: 21063 Score: 70 Expect: 0.0036 Matches: 8 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001264 PE=4 SV=1

	A0A2L1C8U1|A0A2L1C8U1_METMI Mass: 21077 Score: 70 Expect: 0.0036 Matches: 8 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP94_001155 PE=4 SV=1 Q6LZH2|Q6LZH2_METMP Mass: 21063 Score: 70 Expect: 0.0036 Matches: 8 Uncharacterized protein OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=MMP0656 PE=4 SV=1

A3. 21) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no:1 from peak three of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 112 Expect: 2.5e-007 Matches: 14 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1 G0H3B3|G0H3B3_METMI Mass: 61040 Score: 112 Expect: 2.5e-007 Matches: 14 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08645 PE=3 SV=1 A0A2L1CBB0|A0A2L1CBB0_METMI Mass: 61038 Score: 112 Expect: 2.5e-007 Matches: 14 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=mcrA PE=3 SV=1 A0A2Z5PTJ7|A0A2Z5PTJ7_METMI Mass: 61024 Score: 112 Expect: 2.5e-007 Matches: 14 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrA PE=3 SV=1 A0A7J9PKB0|A0A7J9PKB0_METMI Mass: 61040 Score: 112 Expect: 2.5e-007 Matches: 14 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP93_000168 PE=3 SV=1 A0A7J9S9S4|A0A7J9S9S4_METMI Mass: 61024 Score: 112 Expect: 2.5e-007 Matches: 14 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP96_000849 PE=3 SV=1 A0A7J9NQV7|A0A7J9NQV7_METMI Mass: 61031 Score: 112 Expect: 2.5e-007 Matches: 14 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP86_000167 PE=3 SV=1 A0A2Z5PKS6|A0A2Z5PKS6_METMI Mass: 61016 Score: 112 Expect: 2.5e-007 Matches: 14 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrA PE=3 SV=1 A0A7J9S5U7|A0A7J9S5U7_METMI Mass: 61031 Score: 100 Expect: 4.3e-006 Matches: 13 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP92_000701 PE=3 SV=1 A0A7J9NYX4|A0A7J9NYX4_METMI Mass: 60983 Score: 100 Expect: 4.3e-006 Matches: 13 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP87_000167 PE=3 SV=1 A4FVW6|A4FVW6_METM5 Mass: 61055 Score: 67 Expect: 0.0077 Matches: 10 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0017 PE=3 SV=1

A3. 22) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no:2 from peak three of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Q6M0W4|Q6M0W4_METMP Mass: 56311 Score: 202 Expect: 2.5e-016 Matches: 22 UPF0288 protein MMP0154 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=MMP0154 PE=3 SV=1 A0A7J9S6C1|A0A7J9S6C1_METMI Mass: 56324 Score: 185 Expect: 1.2e-014 Matches: 21 UPF0288 protein HNP97_001745 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001745 PE=3 SV=1 A0A7J9NWE6|A0A7J9NWE6_METMI Mass: 56312 Score: 180 Expect: 3.9e-014 Matches: 20 UPF0288 protein HNP86_002165 OS=Methanococcus maripaludis OX=39152 GN=HNP86_002165 PE=3 SV=1 A0A2Z5PIK5|A0A2Z5PIK5_METMI Mass: 56312 Score: 180 Expect: 3.9e-014 Matches: 20 UPF0288 protein MMKA1_01590 OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_01590 PE=3 SV=1 A0A7J9P8K9|A0A7J9P8K9_METMI Mass: 56419 Score: 173 Expect: 2e-013 Matches: 20 UPF0288 protein HNP93_001747 OS=Methanococcus maripaludis OX=39152 GN=HNP93_001747 PE=3 SV=1 A0A7J9NLA3|A0A7J9NLA3_METMI Mass: 56296 Score: 171 Expect: 3.1e-013 Matches: 20 UPF0288 protein HNP88_000130 OS=Methanococcus maripaludis OX=39152 GN=HNP88_000130 PE=3 SV=1 A0A2Z5PUN7|A0A2Z5PUN7_METMI Mass: 56312 Score: 168 Expect: 6.3e-013 Matches: 19 UPF0288 protein MMOS7_01850 OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_01850 PE=3 SV=1 A0A7J9SEK5|A0A7J9SEK5_METMI Mass: 56400 Score: 150 Expect: 3.9e-011 Matches: 18 UPF0288 protein HNP96_001657 OS=Methanococcus maripaludis OX=39152 GN=HNP96_001657 PE=3 SV=1 A0A7J9PNM7|A0A7J9PNM7_METMI Mass: 56370 Score: 150 Expect: 3.9e-011 Matches: 18 UPF0288 protein HNP94_001880 OS=Methanococcus maripaludis OX=39152 GN=HNP94_001880 PE=3 SV=1 A0A2L1CAI0|A0A2L1CAI0_METMI Mass: 56354 Score: 150 Expect: 3.9e-011 Matches: 18 UPF0288 protein MMJJ_09300 OS=Methanococcus maripaludis OX=39152 GN=MMJJ_09300 PE=3 SV=1

	G0H1N9|G0H1N9_METMI Mass: 56282 Score: 140 Expect: 3.9e-010 Matches: 17 UPF0288 protein GYY_00790 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_00790 PE=3 SV=1 A0A7J9PWS2|A0A7J9PWS2_METMI Mass: 56439 Score: 76 Expect: 0.0009 Matches: 12 UPF0288 protein HNP95_001459 OS=Methanococcus maripaludis OX=39152 GN=HNP95_001459 PE=3 SV=1 A0A7J9S5B7|A0A7J9S5B7_METMI Mass: 56440 Score: 76 Expect: 0.0009 Matches: 12 UPF0288 protein HNP89_001284 OS=Methanococcus maripaludis OX=39152 GN=HNP89_001284 PE=3 SV=1 A0A7J9NIT0|A0A7J9NIT0_METMI Mass: 56430 Score: 65 Expect: 0.011 Matches: 11 UPF0288 protein HNP87_001089 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001089 PE=3 SV=1 A0A7J9PC13|A0A7J9PC13_METMI Mass: 56426 Score: 65 Expect: 0.012 Matches: 11 UPF0288 protein HNP91_001071 OS=Methanococcus maripaludis OX=39152 GN=HNP91_001071 PE=3 SV=1

A3. 23) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no:3 from peak three of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	A0A7J9NIS9|A0A7J9NIS9_METMI Mass: 34922 Score: 94 Expect: 1.8e-005 Matches: 7 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001358 PE=4 SV=1 A0A2L1C9H0|A0A2L1C9H0_METMI Mass: 34949 Score: 94 Expect: 1.8e-005 Matches: 7 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=HNP86_001650 PE=4 SV=1 Q6M050|Q6M050_METMP Mass: 34949 Score: 94 Expect: 1.8e-005 Matches: 7 Uncharacterized protein OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=MMP0421 PE=4 SV=1 A0A2Z5PFC4|A0A2Z5PFC4_METMI Mass: 34921 Score: 75 Expect: 0.0013 Matches: 6 Uncharacterized protein OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_04360 PE=4 SV=1 A0A2Z5PQA8|A0A2Z5PQA8_METMI Mass: 34921 Score: 75 Expect: 0.0013 Matches: 6 Uncharacterized protein OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_04400 PE=4 SV=1 A0A7J9S333|A0A7J9S333_METMI Mass: 34936 Score: 70 Expect: 0.0041 Matches: 6 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001461 PE=4 SV=1 A0A7J9PHI7|A0A7J9PHI7_METMI Mass: 34922 Score: 66 Expect: 0.011 Matches: 5 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP90_001125 PE=4 SV=1 A6VJ56|A6VJ56_METM7 Mass: 34922 Score: 66 Expect: 0.011 Matches: 5 Uncharacterized protein OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_1419 PE=4 SV=1 A0A7J9PN18|A0A7J9PN18_METMI Mass: 34814 Score: 65 Expect: 0.012 Matches: 6 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP94_001513 PE=4 SV=1
2	A0A7J9S9F8|A0A7J9S9F8_METMI Mass: 34939 Score: 93 Expect: 2.1e-005 Matches: 8 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP92_000953 PE=4 SV=1

A3. 24) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no:4 from peak three of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Mixture 1 Total score: 133 Expect: 2e-009 Matches: 23 Components (only one family member shown for each component): Q6LWZ7|Q6LWZ7_METMP Mass: 21340 Score: 119 Expect: 5e-008 Matches: 13 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrC PE=4 SV=1 A4FZG7|MTD_METM5 Mass: 29917 Score: 73 Expect: 0.0019 Matches: 11 F420-dependent methylenetetrahydromethanopterin dehydrogenase OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=mtd PE=3 SV=1
2	Q6LWZ7|Q6LWZ7_METMP Mass: 21340 Score: 119 Expect: 5e-008 Matches: 13 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrC PE=4 SV=1 G0H3B1|G0H3B1_METMI Mass: 21340 Score: 119 Expect: 5e-008 Matches: 13 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08635 PE=4 SV=1 A0A2Z5PM12|A0A2Z5PM12_METMI Mass: 21310 Score: 119 Expect: 5e-008 Matches: 13 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrC PE=4 SV=1 A0A2L1CBQ8|A0A2L1CBQ8_METMI Mass: 21340 Score: 119 Expect: 5e-008 Matches: 13 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=mcrC PE=4 SV=1 A0A2Z5PNK7|A0A2Z5PNK7_METMI Mass: 21340 Score: 119 Expect: 5e-008 Matches: 13 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrC PE=4 SV=1 A0A7J9NGE5|A0A7J9NGE5_METMI Mass: 21319 Score: 96 Expect: 1e-005 Matches: 12 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=HNP87_000169 PE=4 SV=1 A4FVW8|A4FVW8_METM5 Mass: 21428 Score: 84 Expect: 0.00018 Matches: 11 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0019 PE=4 SV=1 A0A7J9P9K7|A0A7J9P9K7_METMI Mass: 21292 Score: 70 Expect: 0.0039 Matches: 10 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=HNP89_000172 PE=4 SV=1

	A6UQK3|A6UQK3_METVS Mass: 21354 Score: 59 Expect: 0.05 Matches: 9 Methyl-coenzyme M reductase operon protein C OS=Methanococcus vannielii (strain ATCC 35089 / DSM 1224 / JCM 13029 / OCM 148 / SB) OX=406327 GN=Mevan_0870 PE=4 SV=1 A0A7J9PFQ9|A0A7J9PFQ9_METMI Mass: 21305 Score: 59 Expect: 0.05 Matches: 9 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=HNP90_000387 PE=4 SV=1 A6VHE5|A6VHE5_METM7 Mass: 21305 Score: 59 Expect: 0.05 Matches: 9 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_0804 PE=4 SV=1 A9A9A4|A9A9A4_METM6 Mass: 21368 Score: 59 Expect: 0.05 Matches: 9 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=MmarC6_1113 PE=4 SV=1 P07960|MCRC_METVA Mass: 21354 Score: 59 Expect: 0.05 Matches: 9 Methyl-coenzyme M reductase operon protein C OS=Methanococcus vannielii OX=2187 GN=mcrC PE=4 SV=1
3	A4FZG7|MTD_METM5 Mass: 29917 Score: 73 Expect: 0.0019 Matches: 11 F420-dependent methylenetetrahydromethanopterin dehydrogenase OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=mtd PE=3 SV=1 Q6M099|MTD_METMP Mass: 29888 Score: 63 Expect: 0.02 Matches: 10 F420-dependent methylenetetrahydromethanopterin dehydrogenase OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mtd PE=3 SV=1 A0A7J9S0U0|A0A7J9S0U0_METMI Mass: 29888 Score: 63 Expect: 0.02 Matches: 10 F420-dependent methylenetetrahydromethanopterin dehydrogenase OS=Methanococcus maripaludis OX=39152 GN=mtd PE=3 SV=1 A0A2Z5PG47|A0A2Z5PG47_METMI Mass: 29888 Score: 63 Expect: 0.02 Matches: 10 F420-dependent methylenetetrahydromethanopterin dehydrogenase OS=Methanococcus maripaludis KA1 OX=637914 GN=mtd PE=3 SV=1

A3. 25) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no:5 from peak three of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Q6LWZ7|Q6LWZ7_METMP Mass: 21340 Score: 62 Expect: 0.023 Matches: 4 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrC PE=4 SV=1 A4FVW8|A4FVW8_METM5 Mass: 21428 Score: 62 Expect: 0.023 Matches: 4 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0019 PE=4 SV=1 G0H3B1|G0H3B1_METMI Mass: 21340 Score: 62 Expect: 0.023 Matches: 4 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08635 PE=4 SV=1 A0A2Z5PM12|A0A2Z5PM12_METMI Mass: 21310 Score: 62 Expect: 0.023 Matches: 4 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrC PE=4 SV=1 A0A7J9NGE5|A0A7J9NGE5_METMI Mass: 21319 Score: 62 Expect: 0.023 Matches: 4 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=HNP87_000169 PE=4 SV=1 A0A2L1CBQ8|A0A2L1CBQ8_METMI Mass: 21340 Score: 62 Expect: 0.023 Matches: 4 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=mcrC PE=4 SV=1 A0A2Z5PNK7|A0A2Z5PNK7_METMI Mass: 21340 Score: 62 Expect: 0.023 Matches: 4 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrC PE=4 SV=1

A3. 26) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no:6 from peak three of SEC of McrC (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	A0A7J9NQB0|A0A7J9NQB0_METMI Mass: 21064 Score: 134 Expect: 1.6e-009 Matches: 14 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001731 PE=4 SV=1 A0A2Z5PMC3|A0A2Z5PMC3_METMI Mass: 21063 Score: 119 Expect: 5e-008 Matches: 13 Uncharacterized protein OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_07070 PE=4 SV=1 G0H411|G0H411_METMI Mass: 21063 Score: 119 Expect: 5e-008 Matches: 13 Methanogenesis marker protein 17 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_02990 PE=4 SV=1 A0A2Z5PJQ8|A0A2Z5PJQ8_METMI Mass: 21063 Score: 119 Expect: 5e-008 Matches: 13 Uncharacterized protein OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_07020 PE=4 SV=1 A0A7J9S1E9|A0A7J9S1E9_METMI Mass: 21063 Score: 119 Expect: 5e-008 Matches: 13 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001264 PE=4 SV=1 Q6LZH2|Q6LZH2_METMP Mass: 21063 Score: 119 Expect: 5e-008 Matches: 13 Uncharacterized protein OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=MMP0656 PE=4 SV=1 A0A7J9S5Z5|A0A7J9S5Z5_METMI Mass: 21046 Score: 104 Expect: 1.6e-006 Matches: 12 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP92_001514 PE=4 SV=1 A0A7J9P1C1|A0A7J9P1C1_METMI Mass: 21064 Score: 102 Expect: 2.5e-006 Matches: 12 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP89_001714 PE=4 SV=1 A0A7J9PBM9|A0A7J9PBM9_METMI Mass: 21078 Score: 102 Expect: 2.5e-006 Matches: 12 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP91_001453 PE=4 SV=1 A0A7J9NM23|A0A7J9NM23_METMI Mass: 21049 Score: 100 Expect: 4e-006 Matches: 12 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP88_000689 PE=4 SV=1 A9A7C8|A9A7C8_METM6 Mass: 21078 Score: 99 Expect: 5.4e-006 Matches: 12 Uncharacterized protein OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=MmarC6_0231 PE=4 SV=1

	A0A2L1C8U1|A0A2L1C8U1_METMI Mass: 21077 Score: 89 Expect: 4.6e-005 Matches: 11 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP94_001155 PE=4 SV=1
2	A0A7J9PUS5|A0A7J9PUS5_METMI Mass: 21037 Score: 97 Expect: 8.4e-006 Matches: 12 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP95_001745 PE=4 SV=1
3	A0A7J9NUG4|A0A7J9NUG4_METMI Mass: 21062 Score: 92 Expect: 2.8e-005 Matches: 12 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP86_001066 PE=4 SV=1 A0A7J9P6S6|A0A7J9P6S6_METMI Mass: 21034 Score: 92 Expect: 2.8e-005 Matches: 12 Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP93_001139 PE=4 SV=1

A3. 27) Raw data of MALDI mass spectrometry analysis of Native-Gel band no:1 from purified McrC sample

Accession	Description	Coverage [%]	# AAs	MW [kDa]	Score	Coverage [%]	# Peptides
A0A2L1CBG2	Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=mcrG PE=3 SV=1	55	260	29.6	941	55	15
A0A7J9NLA3	UPF0288 protein HNP88_000130 OS=Methanococcus maripaludis OX=39152 GN=HNP88_000130 PE=3 SV=1	39	501	56.3	793	39	19
A0A2L1CBB9	Methyl-coenzyme M reductase operon protein D OS=Methanococcus maripaludis OX=39152 GN=mcrD PE=4 SV=1	33	159	18.1	169	33	6
A0A2Z5PM12	Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrC PE=4 SV=1	30	198	21.3	594	30	7
Q6LWZ5	Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1	28	553	61	937	28	17
A0A2L1C8U1	Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP94_001155 PE=4 SV=1	26	183	21.1	150	26	5
A0A2L1C9V8	F420-dependent methylenetetrahydromethanopterin dehydrogenase OS=Methanococcus maripaludis OX=39152 GN=mtd PE=3 SV=1	24	277	29.9	331	24	8
Q6M088	S-layer protein OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=slp PE=4 SV=1	23	575	58.9	423	23	13
A0A2L1CBB3	Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=mcB PE=3 SV=1	19	443	46.7	659	19	8
MMPRSt224	Auburn Tagged MM7 OS=Methanococcus maripaludis S2	16	359	40.9	206	16	9
A0A7J9NQ66	Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001691 PE=4 SV=1	12	531	59.5	279	12	8

A3. 28) Raw data of MALDI mass spectrometry analysis of Native-Gel band no:2 from purified McrC sample

Accession	Description	Coverage [%]	# AAs	MW [kDa]	Score	Coverage [%]	# Peptides
A0A2L1CBG2	Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=mcrG PE=3 SV=1	55	260	29.6	941	55	15
A0A7J9NLA3	UPF0288 protein HNP88_000130 OS=Methanococcus maripaludis OX=39152 GN=HNP88_000130 PE=3 SV=1	39	501	56.3	793	39	19
A0A2L1CBB9	Methyl-coenzyme M reductase operon protein D OS=Methanococcus maripaludis OX=39152 GN=mcrD PE=4 SV=1	33	159	18.1	169	33	6
A0A2Z5PM12	Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrC PE=4 SV=1	30	198	21.3	594	30	7
Q6LWZ5	Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1	28	553	61	937	28	17
A0A2L1C8U1	Putative methanogenesis marker protein 17 OS=Methanococcus maripaludis OX=39152 GN=HNP94_001155 PE=4 SV=1	26	183	21.1	150	26	5
A0A2L1C9V8	F420-dependent methylenetetrahydromethanopterin dehydrogenase OS=Methanococcus maripaludis OX=39152 GN=mtd PE=3 SV=1	24	277	29.9	331	24	8
Q6M088	S-layer protein OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=slp PE=4 SV=1	23	575	58.9	423	23	13
A0A2L1CBB3	Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=mcB PE=3 SV=1	19	443	46.7	659	19	8
MMPRSt224	Auburn Tagged MM7 OS=Methanococcus maripaludis S2	16	359	40.9	206	16	9
A0A7J9NQ66	Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001691 PE=4 SV=1	12	531	59.5	279	12	8

A3. 29) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 1 from purified Mmp7 sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	A0A7J9NJY5|A0A7J9NJY5_METMI Mass: 87213 Score: 98 Expect: 6.9e-006 Matches: 21 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP87_001111 PE=3 SV=1 G0H1R1|G0H1R1_METMI Mass: 87160 Score: 97 Expect: 7.6e-006 Matches: 21 Cell division protein CDC48 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_00900 PE=3 SV=1 2::A0A2Z5PFT1|A0A2Z5PFT1_METMI Mass: 87169 Score: 97 Expect: 7.8e-006 Matches: 21 CDC48 cell division cycle protein OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_01810 PE=3 SV=1 A0A2L1CAB7|A0A2L1CAB7_METMI Mass: 87296 Score: 97 Expect: 7.8e-006 Matches: 21 ATP-dependent zinc metalloprotease FtsH 3 OS=Methanococcus maripaludis OX=39152 GN=ftsH3 PE=3 SV=1 A0A7J9NXQ1|A0A7J9NXQ1_METMI Mass: 87183 Score: 97 Expect: 7.8e-006 Matches: 21 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP86_002187 PE=3 SV=1 A0A2Z5PHU0|A0A2Z5PHU0_METMI Mass: 87157 Score: 97 Expect: 7.8e-006 Matches: 21 CDC48 cell division cycle protein OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_02070 PE=3 SV=1 A0A7J9S3B8|A0A7J9S3B8_METMI Mass: 87155 Score: 97 Expect: 7.8e-006 Matches: 21 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP97_001723 PE=3 SV=1 A0A7J9SBX8|A0A7J9SBX8_METMI Mass: 87197 Score: 97 Expect: 7.8e-006 Matches: 21 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP94_001902 PE=3 SV=1 A0A7J9P876|A0A7J9P876_METMI Mass: 87183 Score: 97 Expect: 7.8e-006 Matches: 21 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP88_000152 PE=3 SV=1 Q6M0U2|Q6M0U2_METMP Mass: 87183 Score: 97 Expect: 7.8e-006 Matches: 21 CDC48 cell division cycle protein family member OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=MMP0176 PE=3 SV=1 A0A7J9PBX4|A0A7J9PBX4_METMI Mass: 87244 Score: 76 Expect: 0.00091 Matches: 19 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP91_001093 PE=3 SV=1

	A0A7J9PV30|A0A7J9PV30_METMI Mass: 87228 Score: 76 Expect: 0.00091 Matches: 19 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP89_001262 PE=3 SV=1
2	A9A8B9|A9A8B9_METM6 Mass: 86460 Score: 63 Expect: 0.02 Matches: 17 AAA family ATPase, CDC48 subfamily OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=MmarC6_0775 PE=3 SV=1

A3. 30) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 2 from purified Mmp7 sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 91 Expect: 3e-005 Matches: 11 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1 A0A7J9NQV7|A0A7J9NQV7_METMI Mass: 61031 Score: 91 Expect: 3e-005 Matches: 11 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP86_000167 PE=3 SV=1 G0H3B3|G0H3B3_METMI Mass: 61040 Score: 79 Expect: 0.00047 Matches: 10 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08645 PE=3 SV=1 A0A2L1CBB0|A0A2L1CBB0_METMI Mass: 61038 Score: 79 Expect: 0.00047 Matches: 10 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=mcrA PE=3 SV=1 A0A2Z5PTJ7|A0A2Z5PTJ7_METMI Mass: 61024 Score: 79 Expect: 0.00047 Matches: 10 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrA PE=3 SV=1 A0A7J9PKB0|A0A7J9PKB0_METMI Mass: 61040 Score: 79 Expect: 0.00047 Matches: 10 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP93_000168 PE=3 SV=1 A0A7J9S9S4|A0A7J9S9S4_METMI Mass: 61024 Score: 79 Expect: 0.00047 Matches: 10 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP96_000849 PE=3 SV=1 A0A2Z5PKS6|A0A2Z5PKS6_METMI Mass: 61016 Score: 79 Expect: 0.00047 Matches: 10 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrA PE=3 SV=1 A0A7J9S5U7|A0A7J9S5U7_METMI Mass: 61031 Score: 78 Expect: 0.00063 Matches: 10 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP92_000701 PE=3 SV=1 A0A7J9NYX4|A0A7J9NYX4_METMI Mass: 60983 Score: 78 Expect: 0.00063 Matches: 10 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP87_000167 PE=3 SV=1

A3. 31) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 3 from purified Mmp7 sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	A0A7J9S6C1|A0A7J9S6C1_METMI Mass: 56324 Score: 83 Expect: 0.00019 Matches: 12 UPF0288 protein HNP97_001745 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001745 PE=3 SV=1 Q6M0W4|Q6M0W4_METMP Mass: 56311 Score: 83 Expect: 0.00019 Matches: 12 UPF0288 protein MMP0154 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=MMP0154 PE=3 SV=1 A0A7J9NLA3|A0A7J9NLA3_METMI Mass: 56296 Score: 83 Expect: 0.00021 Matches: 12 UPF0288 protein HNP88_000130 OS=Methanococcus maripaludis OX=39152 GN=HNP88_000130 PE=3 SV=1 A0A7J9P8K9|A0A7J9P8K9_METMI Mass: 56419 Score: 71 Expect: 0.0032 Matches: 11 UPF0288 protein HNP93_001747 OS=Methanococcus maripaludis OX=39152 GN=HNP93_001747 PE=3 SV=1 A0A7J9NWE6|A0A7J9NWE6_METMI Mass: 56312 Score: 65 Expect: 0.014 Matches: 10 UPF0288 protein HNP86_002165 OS=Methanococcus maripaludis OX=39152 GN=HNP86_002165 PE=3 SV=1 A0A2Z5PIK5|A0A2Z5PIK5_METMI Mass: 56312 Score: 65 Expect: 0.014 Matches: 10 UPF0288 protein MMKA1_01590 OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_01590 PE=3 SV=1

A3. 32) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 4 from purified Mmp7 sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	G0H3A9|G0H3A9_METMI Mass: 46617 Score: 97 Expect: 8.7e-006 Matches: 9 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08625 PE=3 SV=1 A0A7J9NPS6|A0A7J9NPS6_METMI Mass: 46617 Score: 97 Expect: 8.7e-006 Matches: 9 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP88_001639 PE=3 SV=1 Q6LWZ9|Q6LWZ9_METMP Mass: 46617 Score: 97 Expect: 8.7e-006 Matches: 9 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrB PE=3 SV=1 A0A2L1CBB3|A0A2L1CBB3_METMI Mass: 46661 Score: 97 Expect: 8.7e-006 Matches: 8 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=mcB PE=3 SV=1 A0A7J9SF28|A0A7J9SF28_METMI Mass: 46631 Score: 97 Expect: 8.7e-006 Matches: 8 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP94_000172 PE=3 SV=1 A0A2Z5PK72|A0A2Z5PK72_METMI Mass: 46670 Score: 80 Expect: 0.00035 Matches: 7 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrB PE=3 SV=1 A0A7J9S0S7|A0A7J9S0S7_METMI Mass: 46661 Score: 80 Expect: 0.00044 Matches: 7 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP97_000800 PE=3 SV=1 A0A7J9NQV4|A0A7J9NQV4_METMI Mass: 46661 Score: 65 Expect: 0.013 Matches: 7 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP86_000171 PE=3 SV=1 A0A2Z5PEG7|A0A2Z5PEG7_METMI Mass: 46615 Score: 65 Expect: 0.014 Matches: 7 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrB PE=3 SV=1 A4FVX0|A4FVX0_METM5 Mass: 46563 Score: 62 Expect: 0.023 Matches: 6 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0021 PE=3 SV=1

A3. 33) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 5 from purified Mmp7 sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	MMPRSt224|MMP_RS02240_METMI Mass: 40900 Score: 67 Expect: 0.0088 Matches: 9 Auburn Tagged MM7 OS=Methanococcus maripaludis S2

A3. 34) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 6 from purified Mmp7 sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	A0A2L1CCB5|A0A2L1CCB5_METMI Mass: 37713 Score: 60 Expect: 0.035 Matches: 6 UPF0219 protein MMJJ_16340 OS=Methanococcus maripaludis OX=39152 GN=fabH PE=3 SV=1

A3. 35) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no: 7 from purified Mmp7 sample (Anaerobic purification) (Protein scores greater than 58 are ($p<0.05$) are shown)

Hits	Concise Protein Summary
1	D7DRD8|D7DRD8_METV3 Mass: 69268 Score: 67 Expect: 0.0081 Matches: 13 Fumarate reductase/succinate dehydrogenase flavoprotein domain protein OS=Methanococcus voltae (strain ATCC BAA-1334 / A3) OX=456320 GN=Mvol_0038 PE=4 SV=1

A3. 36) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE **band no:1 from peak two of SEC** of Mmp7

Hits	Concise Protein Summary
1	A0A7J9NJY5|A0A7J9NJY5_METMI Mass: 87213 Score: 250 Expect: 4.4e-021 Matches: 25 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP87_001111 PE=3 SV=1 G0H1R1|G0H1R1_METMI Mass: 87160 Score: 250 Expect: 4.4e-021 Matches: 25 Cell division protein CDC48 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_00900 PE=3 SV=1 A0A2Z5PFT1|A0A2Z5PFT1_METMI Mass: 87169 Score: 250 Expect: 4.4e-021 Matches: 25 CDC48 cell division cycle protein OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_01810 PE=3 SV=1 A0A2L1CAB7|A0A2L1CAB7_METMI Mass: 87296 Score: 250 Expect: 4.4e-021 Matches: 25 ATP-dependent zinc metalloprotease FtsH 3 OS=Methanococcus maripaludis OX=39152 GN=ftsH3 PE=3 SV=1 A0A7J9NXQ1|A0A7J9NXQ1_METMI Mass: 87183 Score: 250 Expect: 4.4e-021 Matches: 25 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP86_002187 PE=3 SV=1 A0A2Z5PHU0|A0A2Z5PHU0_METMI Mass: 87157 Score: 250 Expect: 4.4e-021 Matches: 25 CDC48 cell division cycle protein OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_02070 PE=3 SV=1 A0A7J9S3B8|A0A7J9S3B8_METMI Mass: 87155 Score: 250 Expect: 4.4e-021 Matches: 25 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP97_001723 PE=3 SV=1 A0A7J9SBX8|A0A7J9SBX8_METMI Mass: 87197 Score: 250 Expect: 4.4e-021 Matches: 25 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP94_001902 PE=3 SV=1 A0A7J9P876|A0A7J9P876_METMI Mass: 87183 Score: 250 Expect: 4.4e-021 Matches: 25 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP88_000152 PE=3 SV=1 Q6M0U2|Q6M0U2_METMP Mass: 87183 Score: 250 Expect: 4.4e-021 Matches: 25 CDC48 cell division cycle protein family member OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=MMP0176 PE=3 SV=1

	A0A7J9PBX4|A0A7J9PBX4_METMI Mass: 87244 Score: 234 Expect: 1.8e-019 Matches: 24 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP91_001093 PE=3 SV=1 A0A7J9PV30|A0A7J9PV30_METMI Mass: 87228 Score: 234 Expect: 1.8e-019 Matches: 24 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP89_001262 PE=3 SV=1 A9A8B9|A9A8B9_METM6 Mass: 86460 Score: 152 Expect: 2.8e-011 Matches: 17 AAA family ATPase, CDC48 subfamily OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=MmarC6_0775 PE=3 SV=1 A0A7J9PIB7|A0A7J9PIB7_METMI Mass: 88852 Score: 120 Expect: 4.4e-008 Matches: 15 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP90_001369 PE=3 SV=1 A6VIG6|A6VIG6_METM7 Mass: 88852 Score: 120 Expect: 4.4e-008 Matches: 15 AAA family ATPase, CDC48 subfamily OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_1176 PE=3 SV=1 A4G012|A4G012_METM5 Mass: 86856 Score: 109 Expect: 5.5e-007 Matches: 14 AAA family ATPase, CDC48 subfamily OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_1499 PE=3 SV=1
2	A6URF6|A6URF6_METVS Mass: 86899 Score: 137 Expect: 8.8e-010 Matches: 17 AAA family ATPase, CDC48 subfamily OS=Methanococcus vannielii (strain ATCC 35089 / DSM 1224 / JCM 13029 / OCM 148 / SB) OX=406327 GN=Mevan_1180 PE=3 SV=1

A3. 37) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no:2 and 3 from peak two of SEC of Mmp7

Hits	Concise Protein Summary
1	Mixture 1 Total score: 150 Expect: 4.4e-011 Matches: 22 Components: A0A7J9S6C1|A0A7J9S6C1_METMI Mass: 56324 Score: 133 Expect: 2.2e-009 Matches: 13 UPF0288 protein HNP97_001745 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001745 PE=3 SV=1 Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 63 Expect: 0.02 Matches: 9 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1
2	Mixture 2 Total score: 149 Expect: 5.5e-011 Matches: 22 Components A0A7J9P8K9|A0A7J9P8K9_METMI Mass: 56419 Score: 132 Expect: 2.8e-009 Matches: 13 UPF0288 protein HNP93_001747 OS=Methanococcus maripaludis OX=39152 GN=HNP93_001747 PE=3 SV=1 Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 63 Expect: 0.02 Matches: 9 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1
3	A0A7J9S6C1|A0A7J9S6C1_METMI Mass: 56324 Score: 133 Expect: 2.2e-009 Matches: 13 UPF0288 protein HNP97_001745 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001745 PE=3 SV=1 Q6M0W4|Q6M0W4_METMP Mass: 56311 Score: 133 Expect: 2.2e-009 Matches: 13 UPF0288 protein MMP0154 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=MMP0154 PE=3 SV=1 A0A7J9NLA3|A0A7J9NLA3_METMI Mass: 56296 Score: 114 Expect: 1.8e-007 Matches: 12 UPF0288 protein HNP88_000130 OS=Methanococcus maripaludis OX=39152 GN=HNP88_000130 PE=3 SV=1 A0A7J9NWE6|A0A7J9NWE6_METMI Mass: 56312 Score: 108 Expect: 7e-007 Matches: 11 UPF0288 protein HNP86_002165 OS=Methanococcus maripaludis OX=39152 GN=HNP86_002165 PE=3 SV=1 A0A2Z5PIK5|A0A2Z5PIK5_METMI Mass: 56312 Score: 108 Expect: 7e-007 Matches: 11 UPF0288 protein MMKA1_01590 OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_01590 PE=3 SV=1 A0A2Z5PUN7|A0A2Z5PUN7_METMI Mass: 56312 Score: 95 Expect: 1.4e-005 Matches: 10 UPF0288 protein MMOS7_01850 OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_01850 PE=3 SV=1

	G0H1N9|G0H1N9_METMI Mass: 56282 Score: 81 Expect: 0.00036 Matches: 9 UPF0288 protein GYY_00790 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_00790 PE=3 SV=1 A0A7J9SEK5|A0A7J9SEK5_METMI Mass: 56400 Score: 78 Expect: 0.00076 Matches: 9 UPF0288 protein HNP96_001657 OS=Methanococcus maripaludis OX=39152 GN=HNP96_001657 PE=3 SV=1 A0A7J9PNM7|A0A7J9PNM7_METMI Mass: 56370 Score: 78 Expect: 0.00076 Matches: 9 UPF0288 protein HNP94_001880 OS=Methanococcus maripaludis OX=39152 GN=HNP94_001880 PE=3 SV=1 A0A2L1CAI0|A0A2L1CAI0_METMI Mass: 56354 Score: 78 Expect: 0.00076 Matches: 9 UPF0288 protein MMJJ_09300 OS=Methanococcus maripaludis OX=39152 GN=MMJJ_09300 PE=3 SV=1
4	A0A7J9P8K9|A0A7J9P8K9_METMI Mass: 56419 Score: 132 Expect: 2.8e-009 Matches: 13 UPF0288 protein HNP93_001747 OS=Methanococcus maripaludis OX=39152 GN=HNP93_001747 PE=3 SV=1
5	Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 63 Expect: 0.02 Matches: 9 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1 G0H3B3|G0H3B3_METMI Mass: 61040 Score: 63 Expect: 0.02 Matches: 9 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08645 PE=3 SV=1 A0A2L1CBB0|A0A2L1CBB0_METMI Mass: 61038 Score: 63 Expect: 0.02 Matches: 9 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=mcrA PE=3 SV=1 A0A2Z5PTJ7|A0A2Z5PTJ7_METMI Mass: 61024 Score: 63 Expect: 0.02 Matches: 9 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrA PE=3 SV=1 A0A7J9PKB0|A0A7J9PKB0_METMI Mass: 61040 Score: 63 Expect: 0.02 Matches: 9 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP93_000168 PE=3 SV=1 A0A7J9S9S4|A0A7J9S9S4_METMI Mass: 61024 Score: 63 Expect: 0.02 Matches: 9 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP96_000849 PE=3 SV=1 A0A7J9NQV7|A0A7J9NQV7_METMI Mass: 61031 Score: 63 Expect: 0.02 Matches: 9 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP86_000167 PE=3 SV=1 A0A2Z5PKS6|A0A2Z5PKS6_METMI Mass: 61016 Score: 63 Expect: 0.02 Matches: 9 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrA PE=3 SV=1

A3. 38) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no:6 from peak **two** of SEC of Mmp7

Hits	Concise Protein Summary
1	MMPRS224|MMP_RS02240_METMI Mass: 34878 Score: 111 Expect: 3.5e-007 Matches: 7 Auburn WO tag MM7 OS=Methanococcus maripaludis S2 A0A7J9NIS9|A0A7J9NIS9_METMI Mass: 34922 Score: 110 Expect: 4.4e-007 Matches: 7 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001358 PE=4 SV=1 A0A2L1C9H0|A0A2L1C9H0_METMI Mass: 34949 Score: 110 Expect: 4.4e-007 Matches: 7 Methyl-coenzyme M reductase operon protein C OS=Methanococcus maripaludis OX=39152 GN=HNP86_001650 PE=4 SV=1 Q6M050|Q6M050_METMP Mass: 34949 Score: 110 Expect: 4.4e-007 Matches: 7 Uncharacterized protein OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=MMP0421 PE=4 SV=1 G0H350|G0H350_METMI Mass: 34921 Score: 110 Expect: 4.4e-007 Matches: 7 Methanogenesis marker protein 7 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_02180 PE=4 SV=1 A0A2Z5PFC4|A0A2Z5PFC4_METMI Mass: 34921 Score: 110 Expect: 4.4e-007 Matches: 7 Uncharacterized protein OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_04360 PE=4 SV=1 A0A2Z5PQA8|A0A2Z5PQA8_METMI Mass: 34921 Score: 110 Expect: 4.4e-007 Matches: 7 Uncharacterized protein OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_04400 PE=4 SV=1 A0A7J9S9F8|A0A7J9S9F8_METMI Mass: 34939 Score: 91 Expect: 3.3e-005 Matches: 6 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP92_000953 PE=4 SV=1 A0A7J9S333|A0A7J9S333_METMI Mass: 34936 Score: 84 Expect: 0.00016 Matches: 6 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001461 PE=4 SV=1 A0A7J9PN18|A0A7J9PN18_METMI Mass: 34814 Score: 80 Expect: 0.00046 Matches: 6 Putative methanogenesis marker protein 7 OS=Methanococcus maripaludis OX=39152 GN=HNP94_001513 PE=4 SV=1

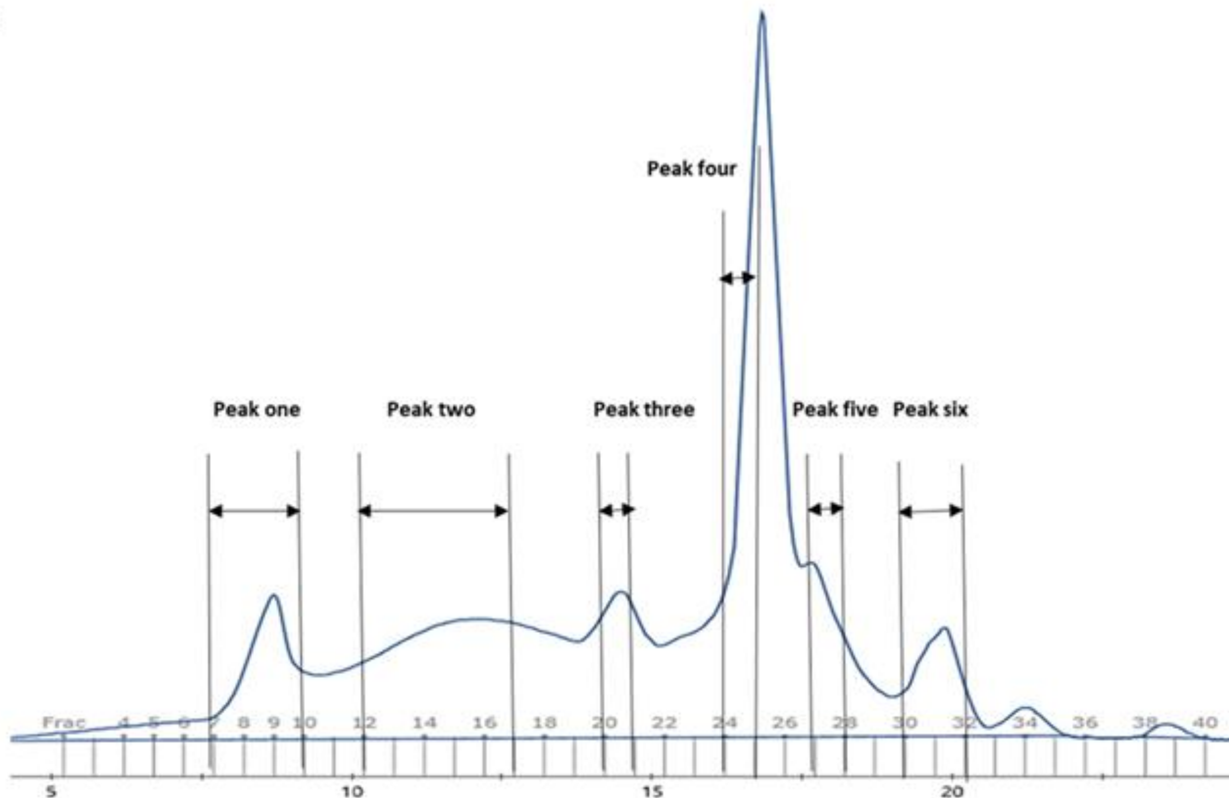
A3. 39) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no:1 from peak three of SEC of Mmp7

Hits	Concise Protein Summary
1	Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 95 Expect: 1.4e-005 Matches: 8 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1 G0H3B3|G0H3B3_METMI Mass: 61040 Score: 95 Expect: 1.4e-005 Matches: 8 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08645 PE=3 SV=1 A0A2L1CBB0|A0A2L1CBB0_METMI Mass: 61038 Score: 95 Expect: 1.4e-005 Matches: 8 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=mcrA PE=3 SV=1 A0A2Z5PTJ7|A0A2Z5PTJ7_METMI Mass: 61024 Score: 95 Expect: 1.4e-005 Matches: 8 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrA PE=3 SV=1 A0A7J9PKB0|A0A7J9PKB0_METMI Mass: 61040 Score: 95 Expect: 1.4e-005 Matches: 8 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP93_000168 PE=3 SV=1 A0A7J9S9S4|A0A7J9S9S4_METMI Mass: 61024 Score: 95 Expect: 1.4e-005 Matches: 8 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP96_000849 PE=3 SV=1 A0A7J9NQV7|A0A7J9NQV7_METMI Mass: 61031 Score: 95 Expect: 1.4e-005 Matches: 8 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP86_000167 PE=3 SV=1 A0A2Z5PKS6|A0A2Z5PKS6_METMI Mass: 61016 Score: 95 Expect: 1.4e-005 Matches: 8 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrA PE=3 SV=1 A0A7J9NYX4|A0A7J9NYX4_METMI Mass: 60983 Score: 77 Expect: 0.00094 Matches: 7 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP87_000167 PE=3 SV=1 A0A7J9S5U7|A0A7J9S5U7_METMI Mass: 61031 Score: 63 Expect: 0.022 Matches: 6 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP92_000701 PE=3 SV=1

A3. 40) Raw data of Mass spectrometry protein fingerprinting analysis of SDS-PAGE band no:2 from peak three of SEC of Mmp7

Hits	Concise Protein Summary
1	Mixture 1 Total score: 188 Expect: 7e-015 Matches: 28 Components: A0A7J9S6C1|A0A7J9S6C1_METMI Mass: 56324 Score: 143 Expect: 2.2e-010 Matches: 17 UPF0288 protein HNP97_001745 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001745 PE=3 SV=1 Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 87 Expect: 8.2e-005 Matches: 11 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1
2	Mixture 2 Total score: 188 Expect: 7e-015 Matches: 28 A0A7J9P8K9|A0A7J9P8K9_METMI Mass: 56419 Score: 142 Expect: 2.8e-010 Matches: 17 UPF0288 protein HNP93_001747 OS=Methanococcus maripaludis OX=39152 GN=HNP93_001747 PE=3 SV=1 Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 87 Expect: 8.2e-005 Matches: 11 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1
3	A0A7J9S6C1|A0A7J9S6C1_METMI Mass: 56324 Score: 143 Expect: 2.2e-010 Matches: 17 UPF0288 protein HNP97_001745 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001745 PE=3 SV=1 Q6M0W4|Q6M0W4_METMP Mass: 56311 Score: 143 Expect: 2.2e-010 Matches: 17 UPF0288 protein MMP0154 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=MMP0154 PE=3 SV=1 A0A7J9NLA3|A0A7J9NLA3_METMI Mass: 56296 Score: 124 Expect: 1.8e-008 Matches: 16 UPF0288 protein HNP88_000130 OS=Methanococcus maripaludis OX=39152 GN=HNP88_000130 PE=3 SV=1

	A0A7J9NWE6|A0A7J9NWE6_METMI Mass: 56312 Score: 119 Expect: 5.5e-008 Matches: 15 UPF0288 protein HNP86_002165 OS=Methanococcus maripaludis OX=39152 GN=HNP86_002165 PE=3 SV=1 A0A2Z5PIK5|A0A2Z5PIK5_METMI Mass: 56312 Score: 119 Expect: 5.5e-008 Matches: 15 UPF0288 protein MMKA1_01590 OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_01590 PE=3 SV=1 A0A2Z5PUN7|A0A2Z5PUN7_METMI Mass: 56312 Score: 106 Expect: 1.1e-006 Matches: 14 UPF0288 protein MMOS7_01850 OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_01850 PE=3 SV=1 G0H1N9|G0H1N9_METMI Mass: 56282 Score: 92 Expect: 2.9e-005 Matches: 13 UPF0288 protein GYY_00790 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_00790 PE=3 SV=1 A0A7J9SEK5|A0A7J9SEK5_METMI Mass: 56400 Score: 88 Expect: 7.1e-005 Matches: 13 UPF0288 protein HNP96_001657 OS=Methanococcus maripaludis OX=39152 GN=HNP96_001657 PE=3 SV=1 A0A7J9PNM7|A0A7J9PNM7_METMI Mass: 56370 Score: 88 Expect: 7.1e-005 Matches: 13 UPF0288 protein HNP94_001880 OS=Methanococcus maripaludis OX=39152 GN=HNP94_001880 PE=3 SV=1 A0A2L1CAI0|A0A2L1CAI0_METMI Mass: 56354 Score: 88 Expect: 7.1e-005 Matches: 13 UPF0288 protein MMJJ_09300 OS=Methanococcus maripaludis OX=39152 GN=MMJJ_09300 PE=3 SV=1
4	A0A7J9P8K9|A0A7J9P8K9_METMI Mass: 56419 Score: 142 Expect: 2.8e-010 Matches: 17 UPF0288 protein HNP93_001747 OS=Methanococcus maripaludis OX=39152 GN=HNP93_001747 PE=3 SV=1
5	Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 87 Expect: 8.2e-005 Matches: 11 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1



A3. 41) FPLC Result for SEC of Mmp7 sample. Eluted Mmp7 sample was purified with Superose (SE) column anaerobically. Peak one (fraction 7-8-9), peak 2 (fraction 12 to 17), peak 3 (fraction 20), peak 4 (fraction 24), peak 5 (fraction 27), and peak 6 (fraction 30-31) were collected, and concentrated with Nanosep Centrifugal Devices with Omega Membrane 10K by centrifugation at 5,000 x g for 20 at room temperature

A3. 42) Raw data of MALDI Protein fingerprinting mass spectrometry analysis of SDS-PAGE band **no: 1** for A2 purified sample

Hits	Concise Protein Summary
1	Mixture 1 Total score: 383 Expect: 2.4e-034 Matches: 77 Components: A0A7J9SBX8|A0A7J9SBX8_METMI Mass: 87197 Score: 226 Expect: 1.2e-018 Matches: 45 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP94_001902 PE=3 SV=1 Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 206 Expect: 1.2e-016 Matches: 40 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1
2	Mixture 2 Total score: 366 Expect: 1.2e-032 Matches: 75 Components A0A7J9SBX8|A0A7J9SBX8_METMI Mass: 87197 Score: 226 Expect: 1.2e-018 Matches: 45 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP94_001902 PE=3 SV=1 A0A7J9NUM7|A0A7J9NUM7_METMI Mass: 59440 Score: 208 Expect: 7.5e-017 Matches: 39 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP86_001138 PE=4 SV=1
3	Mixture 3 Total score: 357 Expect: 9.4e-032 Matches: 75 Components: A0A7J9PBX4|A0A7J9PBX4_METMI Mass: 87244 Score: 208 Expect: 7.5e-017 Matches: 43 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP91_001093 PE=3 SV=1 Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 206 Expect: 1.2e-016 Matches: 40 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1
4	Mixture 4 Total score: 356 Expect: 1.2e-031 Matches: 74 Components: A0A7J9PBX4|A0A7J9PBX4_METMI Mass: 87244 Score: 208 Expect: 7.5e-017 Matches: 43 Transitional endoplasmic reticulum ATPase OS=Methanococcus maripaludis OX=39152 GN=HNP91_001093 PE=3 SV=1 A0A7J9NUM7|A0A7J9NUM7_METMI Mass: 59440 Score: 208 Expect: 7.5e-017 Matches: 39 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP86_001138 PE=4 SV=1

A3. 43)Raw data of MALDI Protein fingerprinting mass spectrometry analysis of SDS-PAGE band **no: 2** for A2 purified sample

Hits	Concise Protein Summary
1	Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 127 Expect: 9.4e-009 Matches: 21 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1 A0A2Z5PV80|A0A2Z5PV80_METMI Mass: 59453 Score: 127 Expect: 9.4e-009 Matches: 21 Methyl coenzyme M reductase system component A2 OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_06650 PE=4 SV=1 G0H4C2|G0H4C2_METMI Mass: 59453 Score: 127 Expect: 9.4e-009 Matches: 21 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_03370 PE=4 SV=1 A0A2L1C9A1|A0A2L1C9A1_METMI Mass: 59453 Score: 127 Expect: 9.4e-009 Matches: 21 Glycine betaine/carnitine/choline transport ATP-binding protein OpuCA OS=Methanococcus maripaludis OX=39152 GN=opuCA PE=4 SV=1 MMP0620|MMP_0620_METMI Mass: 59453 Score: 127 Expect: 9.4e-009 Matches: 21 Auburn recombinant component2 OS=Methanococcus maripaludis S2 3::A0A2Z5PQI8|A0A2Z5PQI8_METMI Mass: 59425 Score: 117 Expect: 9.4e-008 Matches: 20 Methyl coenzyme M reductase system component A2 OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_06700 PE=4 SV=1 A0A7J9NUM7|A0A7J9NUM7_METMI Mass: 59440 Score: 115 Expect: 1.5e-007 Matches: 20 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP86_001138 PE=4 SV=1 A0A7J9RZV0|A0A7J9RZV0_METMI Mass: 59426 Score: 115 Expect: 1.5e-007 Matches: 20 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001226 PE=4 SV=1 A0A7J9NQ66|A0A7J9NQ66_METMI Mass: 59451 Score: 104 Expect: 1.9e-006 Matches: 18 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001691 PE=4 SV=1 A0A7J9PBQ6|A0A7J9PBQ6_METMI Mass: 59437 Score: 104 Expect: 1.9e-006 Matches: 18 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP91_001493 PE=4 SV=1 A0A7J9P287|A0A7J9P287_METMI Mass: 59437 Score: 104 Expect: 1.9e-006 Matches: 18 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP89_001673 PE=4 SV=1

	A0A7J9PUV4|A0A7J9PUV4_METMI Mass: 59411 Score: 104 Expect: 1.9e-006 Matches: 18 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP92_001556 PE=4 SV=1
2	A6VJS5|A6VJS5_METM7 Mass: 59377 Score: 95 Expect: 1.5e-005 Matches: 19 ABC transporter related OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_1643 PE=4 SV=1 A0A7J9PJ23|A0A7J9PJ23_METMI Mass: 59377 Score: 95 Expect: 1.5e-005 Matches: 19 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP90_001663 PE=4 SV=1
3	A4FYK7|A4FYK7_METM5 Mass: 59300 Score: 84 Expect: 0.0002 Matches: 16 ABC transporter related protein OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0985 PE=4 SV=1
4	A9A6A4|A9A6A4_METM6 Mass: 59413 Score: 78 Expect: 0.00068 Matches: 17 Methyl coenzyme M reductase system, component A2 OS=Methanococcus maripaludis (strain C6 / ATCC BAA-1332) OX=444158 GN=MmarC6_0271 PE=4 SV=1

A3. 44) Raw data of MALDI Protein fingerprinting mass spectrometry analysis of SDS-PAGE band **no: 3** for A2 purified sample

Hits	Concise Protein Summary
1	Mixture 1 Total score: 309 Expect: 5.9e-027 Matches: 63 Components: Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 223 Expect: 2.4e-018 Matches: 43 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1 Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 114 Expect: 1.9e-007 Matches: 22 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1
2	Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 223 Expect: 2.4e-018 Matches: 43 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1 A0A2Z5PV80|A0A2Z5PV80_METMI Mass: 59453 Score: 223 Expect: 2.4e-018 Matches: 43 Methyl coenzyme M reductase system component A2 OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_06650 PE=4 SV=1 G0H4C2|G0H4C2_METMI Mass: 59453 Score: 223 Expect: 2.4e-018 Matches: 43 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_03370 PE=4 SV=1 A0A2L1C9A1|A0A2L1C9A1_METMI Mass: 59453 Score: 223 Expect: 2.4e-018 Matches: 43 Glycine betaine/carnitine/choline transport ATP-binding protein OpuCA OS=Methanococcus maripaludis OX=39152 GN=opuCA PE=4 SV=1 A0A7J9NUM7|A0A7J9NUM7_METMI Mass: 59440 Score: 188 Expect: 7.5e-015 Matches: 40 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP86_001138 PE=4 SV=1 A0A7J9RZV0|A0A7J9RZV0_METMI Mass: 59426 Score: 188 Expect: 7.5e-015 Matches: 40 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001226 PE=4 SV=1 A0A7J9NQ66|A0A7J9NQ66_METMI Mass: 59451 Score: 165 Expect: 1.5e-012 Matches: 35 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001691 PE=4 SV=1 A0A7J9PBQ6|A0A7J9PBQ6_METMI Mass: 59437 Score: 165 Expect: 1.5e-012 Matches: 35 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP91_001493 PE=4 SV=1

	A0A7J9P287|A0A7J9P287_METMI Mass: 59437 Score: 165 Expect: 1.5e-012 Matches: 35 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP89_001673 PE=4 SV=1 A0A7J9PUV4|A0A7J9PUV4_METMI Mass: 59411 Score: 165 Expect: 1.5e-012 Matches: 35 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP92_001556 PE=4 SV=1
3	Q6LWZ5|Q6LWZ5_METMP Mass: 61031 Score: 114 Expect: 1.9e-007 Matches: 22 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrA PE=3 SV=1 A0A7J9NQV7|A0A7J9NQV7_METMI Mass: 61031 Score: 114 Expect: 1.9e-007 Matches: 22 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP86_000167 PE=3 SV=1 G0H3B3|G0H3B3_METMI Mass: 61040 Score: 104 Expect: 1.9e-006 Matches: 21 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08645 PE=3 SV=1 A0A2L1CBB0|A0A2L1CBB0_METMI Mass: 61038 Score: 104 Expect: 1.9e-006 Matches: 21 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=mcrA PE=3 SV=1 A0A2Z5PTJ7|A0A2Z5PTJ7_METMI Mass: 61024 Score: 104 Expect: 1.9e-006 Matches: 21 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrA PE=3 SV=1 A0A7J9PKB0|A0A7J9PKB0_METMI Mass: 61040 Score: 104 Expect: 1.9e-006 Matches: 21 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP93_000168 PE=3 SV=1 A0A7J9S9S4|A0A7J9S9S4_METMI Mass: 61024 Score: 104 Expect: 1.9e-006 Matches: 21 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP96_000849 PE=3 SV=1 A0A2Z5PKS6|A0A2Z5PKS6_METMI Mass: 61016 Score: 104 Expect: 1.9e-006 Matches: 21 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrA PE=3 SV=1 A0A7J9S5U7|A0A7J9S5U7_METMI Mass: 61031 Score: 103 Expect: 2.4e-006 Matches: 19 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP92_000701 PE=3 SV=1 A0A7J9NYX4|A0A7J9NYX4_METMI Mass: 60983 Score: 103 Expect: 2.4e-006 Matches: 20 Methyl-coenzyme M reductase subunit alpha OS=Methanococcus maripaludis OX=39152 GN=HNP87_000167 PE=3 SV=1 H7CHX3|H7CHX3_METMI Mass: 41475 Score: 56 Expect: 0.13 Matches: 12 Coenzyme-B sulfoethylthiotransferase (Fragment) OS=Methanococcus maripaludis OX=39152 GN=mcrA PE=3 SV=1

	H7CHX4|H7CHX4_METMI Mass: 40899 Score: 47 Expect: 0.88 Matches: 11 Coenzyme-B sulfoethylthiotransferase (Fragment) OS=Methanococcus maripaludis OX=39152 GN=mcrA PE=3 SV=1
4	A6VJS5|A6VJS5_METM7 Mass: 59377 Score: 92 Expect: 2.7e-005 Matches: 27 ABC transporter related OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_1643 PE=4 SV=1 A0A7J9PJ23|A0A7J9PJ23_METMI Mass: 59377 Score: 92 Expect: 2.7e-005 Matches: 27 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP90_001663 PE=4 SV=1
5	A4FYK7|A4FYK7_METM5 Mass: 59300 Score: 90 Expect: 4.3e-005 Matches: 28 ABC transporter related protein OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0985 PE=4 SV=1

A3. 45) Raw data of MALDI Protein fingerprinting mass spectrometry analysis of SDS-PAGE band **no: 4** for A2 purified sample

Hits	Concise Protein Summary
1	Mixture 1 Total score: 186 Expect: 1.2e-014 Matches: 62 Components: Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 146 Expect: 1.2e-010 Matches: 39 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1 G0H3A9|G0H3A9_METMI Mass: 46617 Score: 81 Expect: 0.0004 Matches: 15 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08625 PE=3 SV=1 Q8TRK9_METAC Mass: 28648 Score: 51 Expect: 0.35 Matches: 12 Uncharacterized protein OS=Methanosarcina acetivorans (strain ATCC 35395 / DSM 2834 / JCM 12185 / C2A) OX=188937 GN=MA_1166 PE=4 SV=1
2	Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 146 Expect: 1.2e-010 Matches: 39 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1 A0A2Z5PV80_METMI Mass: 59453 Score: 146 Expect: 1.2e-010 Matches: 39 Methyl coenzyme M reductase system component A2 OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_06650 PE=4 SV=1 G0H4C2|G0H4C2_METMI Mass: 59453 Score: 146 Expect: 1.2e-010 Matches: 39 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_03370 PE=4 SV=1 A0A2L1C9A1|A0A2L1C9A1_METMI Mass: 59453 Score: 146 Expect: 1.2e-010 Matches: 39 Glycine betaine/carnitine/choline transport ATP-binding protein OpuCA OS=Methanococcus maripaludis OX=39152 GN=opuCA PE=4 SV=1 A0A7J9NQ66|A0A7J9NQ66_METMI Mass: 59451 Score: 108 Expect: 7.5e-007 Matches: 32 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001691 PE=4 SV=1 A0A7J9PBQ6|A0A7J9PBQ6_METMI Mass: 59437 Score: 108 Expect: 7.5e-007 Matches: 32 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP91_001493 PE=4 SV=1 A0A7J9P287|A0A7J9P287_METMI Mass: 59437 Score: 108 Expect: 7.5e-007 Matches: 32 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP89_001673 PE=4 SV=1

	A0A7J9PUV4|A0A7J9PUV4_METMI Mass: 59411 Score: 108 Expect: 7.5e-007 Matches: 32 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP92_001556 PE=4 SV=1
3	A0A7J9NUM7|A0A7J9NUM7_METMI Mass: 59440 Score: 132 Expect: 3e-009 Matches: 36 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP86_001138 PE=4 SV=1 A0A7J9RZV0|A0A7J9RZV0_METMI Mass: 59426 Score: 132 Expect: 3e-009 Matches: 36 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001226 PE=4 SV=1
4	Mixture 2 Total score: 97 Expect: 1e-005 Matches: 38 Components: G0H3A9|G0H3A9_METMI Mass: 46617 Score: 81 Expect: 0.0004 Matches: 15 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08625 PE=3 SV=1 A4FYK7|A4FYK7_METM5 Mass: 59300 Score: 63 Expect: 0.022 Matches: 24 ABC transporter related protein OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0985 PE=4 SV=1
5	Mixture 3 Total score: 90 Expect: 4.3e-005 Matches: 48 Components: A4FYK7|A4FYK7_METM5 Mass: 59300 Score: 63 Expect: 0.022 Matches: 24 ABC transporter related protein OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0985 PE=4 SV=1 A0A2Z5PK72|A0A2Z5PK72_METMI Mass: 46670 Score: 62 Expect: 0.032 Matches: 13 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrB PE=3 SV=1 Q8TRK9_METAC Mass: 28648 Score: 51 Expect: 0.35 Matches: 12 Uncharacterized protein OS=Methanosarcina acetivorans (strain ATCC 35395 / DSM 2834 / JCM 12185 / C2A) OX=188937 GN=MA_1166 PE=4 SV=1
6	G0H3A9|G0H3A9_METMI Mass: 46617 Score: 81 Expect: 0.0004 Matches: 15 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08625 PE=3 SV=1 A0A2L1CBB3|A0A2L1CBB3_METMI Mass: 46661 Score: 81 Expect: 0.0004 Matches: 15 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=mcB PE=3 SV=1 A0A7J9SF28|A0A7J9SF28_METMI Mass: 46631 Score: 81 Expect: 0.0004 Matches: 15 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP94_000172 PE=3 SV=1

	A0A7J9NPS6|A0A7J9NPS6_METMI Mass: 46617 Score: 81 Expect: 0.0004 Matches: 15 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP88_001639 PE=3 SV=1 Q6LWZ9|Q6LWZ9_METMP Mass: 46617 Score: 81 Expect: 0.0004 Matches: 15 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrB PE=3 SV=1 A0A7J9NQV4|A0A7J9NQV4_METMI Mass: 46661 Score: 64 Expect: 0.018 Matches: 13 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP86_000171 PE=3 SV=1 A0A7J9S0S7|A0A7J9S0S7_METMI Mass: 46661 Score: 64 Expect: 0.018 Matches: 13 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OX=39152 GN=HNP97_000800 PE=3 SV=1 A0A2Z5PK72|A0A2Z5PK72_METMI Mass: 46670 Score: 62 Expect: 0.032 Matches: 13 Methyl-coenzyme M reductase subunit beta OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrB PE=3 SV=1

A3. 46) Raw data of MALDI Protein fingerprinting mass spectrometry analysis of SDS-PAGE band **no: 5** for A2 purified sample

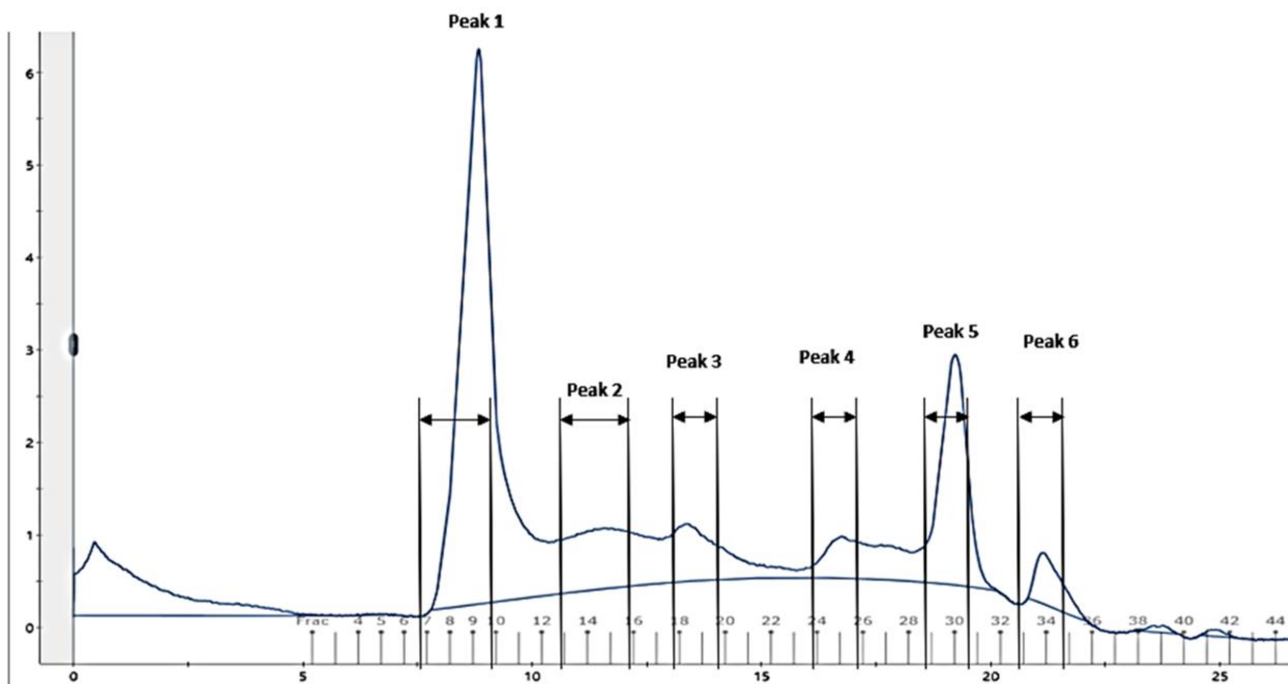
Hits	Concise Protein Summary
1	Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 223 Expect: 2.4e-018 Matches: 42 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1 A0A2Z5PV80|A0A2Z5PV80_METMI Mass: 59453 Score: 223 Expect: 2.4e-018 Matches: 42 Methyl coenzyme M reductase system component A2 OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_06650 PE=4 SV=1 G0H4C2|G0H4C2_METMI Mass: 59453 Score: 223 Expect: 2.4e-018 Matches: 42 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_03370 PE=4 SV=1 A0A2L1C9A1|A0A2L1C9A1_METMI Mass: 59453 Score: 223 Expect: 2.4e-018 Matches: 42 Glycine betaine/carnitine/choline transport ATP-binding protein OpuCA OS=Methanococcus maripaludis OX=39152 GN=opuCA PE=4 SV=1 MMP0620|MMP_0620_METMI Mass: 59453 Score: 223 Expect: 2.4e-018 Matches: 42 Auburn recombinant component2 OS=Methanococcus maripaludis S2 3::A0A2Z5PQI8|A0A2Z5PQI8_METMI Mass: 59425 Score: 205 Expect: 1.5e-016 Matches: 41 Methyl coenzyme M reductase system component A2 OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_06700 PE=4 SV=1 A0A7J9RZV0|A0A7J9RZV0_METMI Mass: 59426 Score: 174 Expect: 1.9e-013 Matches: 37 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001226 PE=4 SV=1 A0A7J9NQ66|A0A7J9NQ66_METMI Mass: 59451 Score: 150 Expect: 4.7e-011 Matches: 34 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001691 PE=4 SV=1 A0A7J9PBQ6|A0A7J9PBQ6_METMI Mass: 59437 Score: 150 Expect: 4.7e-011 Matches: 34 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP91_001493 PE=4 SV=1 A0A7J9P287|A0A7J9P287_METMI Mass: 59437 Score: 150 Expect: 4.7e-011 Matches: 34 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP89_001673 PE=4 SV=1 A0A7J9PUV4|A0A7J9PUV4_METMI Mass: 59411 Score: 150 Expect: 4.7e-011 Matches: 34 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP92_001556 PE=4 SV=1
2	A0A7J9NUM7|A0A7J9NUM7_METMI Mass: 59440 Score: 174 Expect: 1.9e-013 Matches: 38 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP86_001138 PE=4 SV=1

3	A4FYK7|A4FYK7_METM5 Mass: 59300 Score: 96 Expect: 1.3e-005 Matches: 29 ABC transporter related protein OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0985 PE=4 SV=1
4	A6VJS5|A6VJS5_METM7 Mass: 59377 Score: 91 Expect: 3.7e-005 Matches: 27 ABC transporter related OS=Methanococcus maripaludis (strain C7 / ATCC BAA-1331) OX=426368 GN=MmarC7_1643 PE=4 SV=1 A0A7J9PJ23|A0A7J9PJ23_METMI Mass: 59377 Score: 91 Expect: 3.7e-005 Matches: 27 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP90_001663 PE=4 SV=1

A3. 47) Raw data of MALDI Protein fingerprinting mass spectrometry analysis of SDS-PAGE **band no: 6** for A2 purified sample

Hits	Concise Protein Summary
1	Mixture 1 Total score: 201 Expect: 3.7e-016 Matches: 39 Components: 3::A0A7J9NUM7|A0A7J9NUM7_METMI Mass: 59440 Score: 141 Expect: 3.7e-010 Matches: 23 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP86_001138 PE=4 SV=1 A0A7J9NRP7|A0A7J9NRP7_METMI Mass: 29587 Score: 93 Expect: 2.6e-005 Matches: 17 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP86_000168 PE=3 SV=1
2	Mixture 2 Total score: 197 Expect: 9.4e-016 Matches: 39 Components: Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 138 Expect: 7.5e-010 Matches: 23 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1 A0A7J9NRP7|A0A7J9NRP7_METMI Mass: 29587 Score: 93 Expect: 2.6e-005 Matches: 17 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP86_000168 PE=3 SV=1
3	A0A7J9NUM7|A0A7J9NUM7_METMI Mass: 59440 Score: 141 Expect: 3.7e-010 Matches: 23 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP86_001138 PE=4 SV=1 A0A7J9RZV0|A0A7J9RZV0_METMI Mass: 59426 Score: 124 Expect: 1.9e-008 Matches: 22 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001226 PE=4 SV=1
4	Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 138 Expect: 7.5e-010 Matches: 23 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1 A0A2Z5PV80|A0A2Z5PV80_METMI Mass: 59453 Score: 138 Expect: 7.5e-010 Matches: 23 Methyl coenzyme M reductase system component A2 OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_06650 PE=4 SV=1 G0H4C2|G0H4C2_METMI Mass: 59453 Score: 138 Expect: 7.5e-010 Matches: 23 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_03370 PE=4 SV=1 A0A2L1C9A1|A0A2L1C9A1_METMI Mass: 59453 Score: 138 Expect: 7.5e-010 Matches: 23 Glycine betaine/carnitine/choline transport ATP-binding protein OpuCA OS=Methanococcus maripaludis OX=39152 GN=opuCA PE=4 SV=1

5	Mixture 3 Total score: 120 Expect: 4.7e-008 Matches: 32 Components: A0A7J9NRP7|A0A7J9NRP7_METMI Mass: 29587 Score: 93 Expect: 2.6e-005 Matches: 17 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP86_000168 PE=3 SV=1 A4FYK7|A4FYK7_METM5 Mass: 59300 Score: 77 Expect: 0.00098 Matches: 17 ABC transporter related protein OS=Methanococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0985 PE=4 SV=1
6	Mixture 3 Total score: 120 Expect: 4.7e-008 Matches: 32 Components A0A7J9NRP7|A0A7J9NRP7_METMI Mass: 29587 Score: 93 Expect: 2.6e-005 Matches: 17 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP86_000168 PE=3 SV=1 A4FYK7|A4FYK7_METM5 Mass: 59300 Score: 77 Expect: 0.00098 Matches: 17 ABC transporter related protein OS=Meth anococcus maripaludis (strain C5 / ATCC BAA-1333) OX=402880 GN=MmarC5_0985 PE=4 SV=1



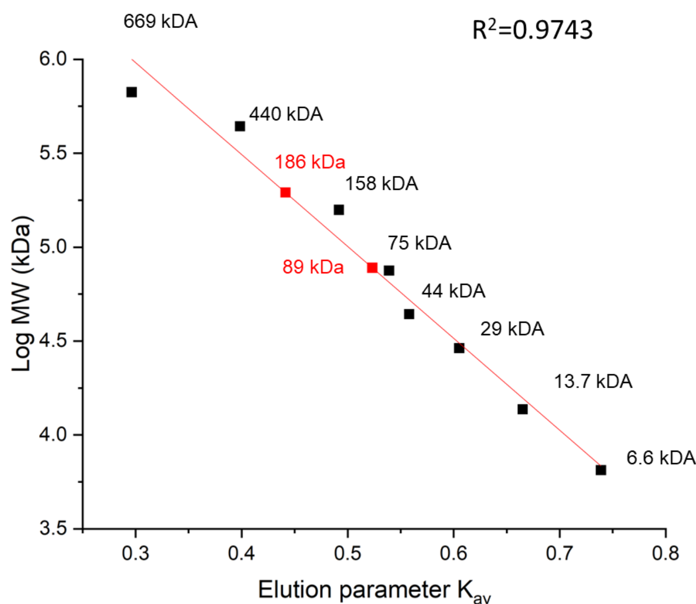
A3. 48) FPLC result for SEC of A2 protein sample. Peak one (fraction 7-8-9), peak 2 (fraction 13-14- 15), peak 3 (fraction 18-19), peak 4 (fraction 24-25), peak 5 (fraction 29-30), and peak 6 (fraction 33-34) were collected and concentrated with Nanosep® Centrifugal Devices with Omega™ Membrane 10K by centrifugation at 5,000 x g for 20 at room temperature

A3. 49) Raw data of MALDI Protein fingerprinting mass spectrometry analysis of SDS-PAGE band no: 2 of peak 2 for SEC of A2 purified sample

Hits	Concise Protein Summary
1	Q6LZK7|Q6LZK7_METMP Mass: 59453 Score: 159 Expect: 5.5e-012 Matches: 29 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=atwA PE=4 SV=1 A0A2Z5PV80|A0A2Z5PV80_METMI Mass: 59453 Score: 159 Expect: 5.5e-012 Matches: 29 Methyl coenzyme M reductase system component A2 OS=Methanococcus maripaludis OS7 OX=637915 GN=MMOS7_06650 PE=4 SV=1 G0H4C2|G0H4C2_METMI Mass: 59453 Score: 159 Expect: 5.5e-012 Matches: 29 Methyl coenzyme M reductase, component A2 OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_03370 PE=4 SV=1 A0A2L1C9A1|A0A2L1C9A1_METMI Mass: 59453 Score: 159 Expect: 5.5e-012 Matches: 29 Glycine betaine/carnitine/choline transport ATP-binding protein OpuCA OS=Methanococcus maripaludis OX=39152 GN=opuCA PE=4 SV=1 MMP0620|MMP_0620_METMI Mass: 59453 Score: 159 Expect: 5.5e-012 Matches: 29 Auburn recombinant component2 OS=Methanococcus maripaludis S2 2::A0A2Z5PQI8|A0A2Z5PQI8_METMI Mass: 59425 Score: 148 Expect: 7e-011 Matches: 28 Methyl coenzyme M reductase system component A2 OS=Methanococcus maripaludis KA1 OX=637914 GN=MMKA1_06700 PE=4 SV=1 A0A7J9NUM7|A0A7J9NUM7_METMI Mass: 59440 Score: 134 Expect: 1.8e-009 Matches: 27 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP86_001138 PE=4 SV=1 A0A7J9RZV0|A0A7J9RZV0_METMI Mass: 59426 Score: 134 Expect: 1.8e-009 Matches: 27 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP97_001226 PE=4 SV=1 A0A7J9NQ66|A0A7J9NQ66_METMI Mass: 59451 Score: 109 Expect: 5.5e-007 Matches: 24 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001691 PE=4 SV=1 A0A7J9PBQ6|A0A7J9PBQ6_METMI Mass: 59437 Score: 109 Expect: 5.5e-007 Matches: 24 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP91_001493 PE=4 SV=1 A0A7J9P287|A0A7J9P287_METMI Mass: 59437 Score: 109 Expect: 5.5e-007 Matches: 24 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP89_001673 PE=4 SV=1

	A0A7J9PUV4|A0A7J9PUV4_METMI Mass: 59411 Score: 109 Expect: 5.5e-007 Matches: 24 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP92_001556 PE=4 SV=1
2	Components A0A7J9NQ66|A0A7J9NQ66_METMI Mass: 59451 Score: 109 Expect: 5.5e-007 Matches: 24 Methyl coenzyme M reductase system subunit A2 OS=Methanococcus maripaludis OX=39152 GN=HNP87_001691 PE=4 SV=1 0A7J9NRP7|A0A7J9NRP7_METMI Mass: 29587 Score: 62 Expect: 0.031 Matches: 13 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP86_000168 PE=3 SV=1
3	A6USB5|A6USB5_METVS Mass: 59530 Score: 62 Expect: 0.025 Matches: 19 ABC transporter related OS=Methanococcus vannielii (strain ATCC 35089 / DSM 1224 / JCM 13029 / OCM 148 / SB) OX=406327 GN=Mevan_1493 PE=4 SV=1
4	A0A7J9NRP7|A0A7J9NRP7_METMI Mass: 29587 Score: 62 Expect: 0.031 Matches: 13 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP86_000168 PE=3 SV=1 Q6LWZ6|Q6LWZ6_METMP Mass: 29587 Score: 62 Expect: 0.031 Matches: 13 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis (strain S2 / LL) OX=267377 GN=mcrG PE=3 SV=1 G0H3B2|G0H3B2_METMI Mass: 29718 Score: 52 Expect: 0.3 Matches: 12 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis X1 OX=1053692 GN=GYY_08640 PE=3 SV=1 A0A2Z5PN58|A0A2Z5PN58_METMI Mass: 29649 Score: 52 Expect: 0.31 Matches: 12 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OS7 OX=637915 GN=mcrG PE=3 SV=1 A0A7J9RZ90|A0A7J9RZ90_METMI Mass: 29619 Score: 52 Expect: 0.31 Matches: 12 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis OX=39152 GN=HNP93_000169 PE=3 SV=1 A0A2Z5PJC7|A0A2Z5PJC7_METMI Mass: 29633 Score: 51 Expect: 0.38 Matches: 12 Methyl-coenzyme M reductase subunit gamma OS=Methanococcus maripaludis KA1 OX=637914 GN=mcrG PE=3 SV=1

Standard curve for McrC



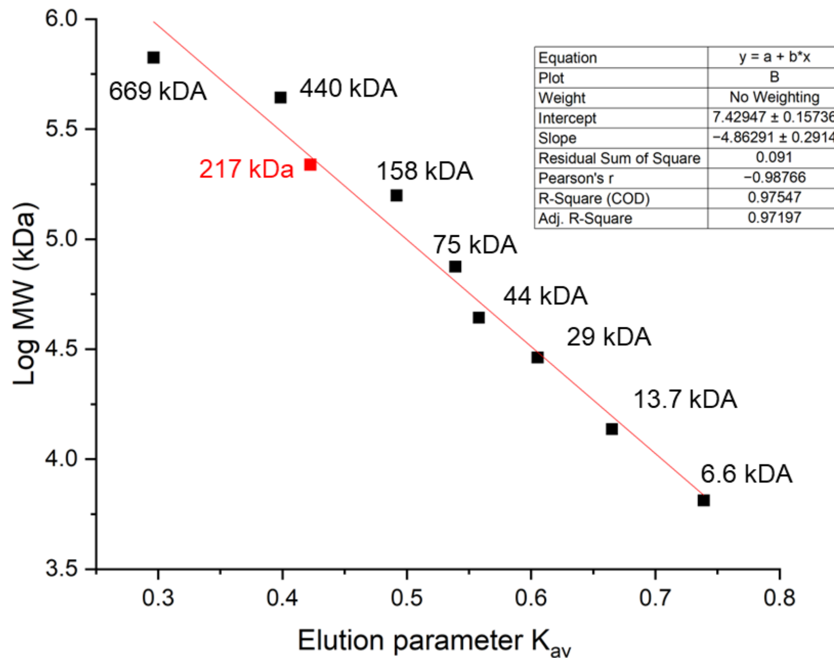
V_0 = void volume (7.7 ml)

V_e = elution volume

V_{col} = Total column volume (23.56 ml)

A3. 50) Standard curve prodcued from a linear fit of the log molecular weight of the protein standards versus their elution parameter Kav= $(V_e - V_0)/(V_{col} - V_0)$. For SEC of **McrC**, estimated molecular weight of eluted protein sample in Peak 2 and 3 were 189 and 86 kD respectively. The standard proteins are Thyroglobulin (Mr 669 000), Ferritin (Mr 440 000), Aldolase (Mr 158 000), Conalbumin (Mr 75 000), Carbonic anhydrase (Mr 29 000), Ribonuclease A (Mr 13 700) and Aprotinin (Mr 6500).

Standard curve for Mmp7



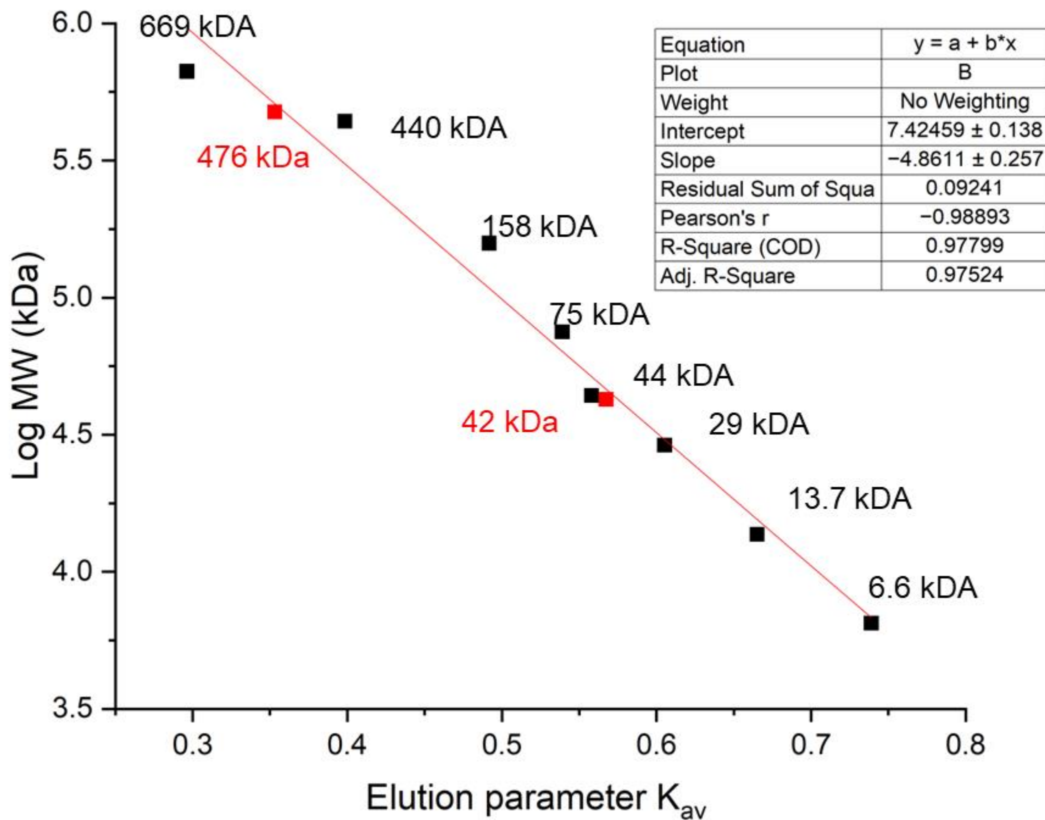
V_0 = void volume (7.7 ml)

V_e = elution volume

V_{col} = Total column volume (23.56 ml)

A3. 51) Standard curve prodcued from a linear fit of the log molecular weight of the protein standards versus their elution parameter $K_{av} = (V_e - V_0)/(V_{col} - V_0)$. For SEC of **Mmp7**, estimated molecular weight of eluted protein sample in Peak 2 and 3 were 1198 and 217 kD respectively. The standard proteins are Thyroglobulin (Mr 669 000), Ferritin (Mr 440 000), Aldolase (Mr 158 000), Conalbumin (Mr 75 000), Carbonic anhydrase (Mr 29 000), Ribonuclease A (Mr 13 700) and Aprotinin (Mr 6500).

Standard curve for A2



A3. 52) Standard curve prodcued from a linear fit of the log molecular weight of the protein standards versus their elution parameter $K_{av} = (V_e - V_0)/(V_{col} - V_0)$. For SEC of **A2**, estimated molecular weight of eluted protein sample in Peak 2, 3 and 4 were 1592, 476, 42 kD respectively. The standard proteins are Thyroglobulin (Mr 669 000), Ferritin (Mr 440 000), Aldolase (Mr 158 000), Conalbumin (Mr 75 000), Carbonic anhydrase (Mr 29 000), Ribonuclease A (Mr 13 700) and Aprotinin (Mr 6500).

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