Towards understanding of copper dependent regulation of soluble methane monooxygenase in *Methylococcus capsulatus* (Bath)

Ph.D. thesis

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INTRODUCTION

Methane, although present in a relatively small amount, has had a central role in many of the main evolutionary events. Also, the influence of methane in global warming should not be understated. The methanotrophic bacteria, a subset of the methylotrophs, are one of the largest biological sinks for methane in aerobic soils and in fresh waters, they have the distinctive property of utilising methane as their sole source of carbon and energy.

The key enzyme in methane metabolism is methane monooxygenase (MMO), which catalyses the oxidation of methane to methanol. Some methanotrophs, including Methylococcus capsulatus (Bath), possess two distinct MMOs. The biosynthesis of the MMO enzymes in these methanotrophs is regulated by the level of copper available to the cells. When methanotrophs are grown at a high copper-to-biomass ratio, the membrane bound, particulate MMO (pMMO) is synthesised. pMMO is present in all known methanotrophs. At a low copper-to-biomass ratio, some methanotrophs produce a soluble, cytoplasmic methane-oxidising enzyme (sMMO), e.g., *Methylococcus capsulatus* (Bath)

A completely copper-deficient environment, which would favor the expression of sMMO, rarely exists in nature therefore pMMO is likely to be functional in most natural environments. This is disadvantageous for potential bioremediation applications since sMMO has much wider substrate specificity than pMMO. sMMO is capable of degrading a number of recalcitrant and carcinogenic hazardous chemicals, hence for biotechnological exploitation, control over the copper-regulated expression of MMOs would be desirable. The amounts of copper ions in the enviroment of the cells also regulate a number of other processes such as biosynthesis of internal membranes and expression of at least two formaldehyde dehydrogenases, etc.

Regulation of the copper-dependent MMO expression takes place at the level of transcription. The intriguing and strong copper regulation of the expression of the MMO enzymes has been the subject of several studies, but details of the mechanism of regulation remain to be elucidated. Previous analysis of the transcription of the *smmo* genes in *M. capsulatus* (Bath) has revealed that the transcription of the *smmo* genes starts from a single promoter upstream (5`) from *mmoX*. In the *mmoX* promoter region, sequences resembling both σ^{70} and a σ^{N} binding sites were identified and a σ^{70}-type promoter was predicted to be the likely promoter.

AIMS

Unique catalytic activity of soluble methane monooxygenases makes them ideal candidates for environmental friendly catalyst for biotechnological processes. The copper sensitivity of sMMO and the fact that it is expressed under copper depleted conditions blocks the development of biotechnological applications. Detailed knowledge of copper dependent soluble methane monooxygenase regulation would be desirable to promote the development of environmental friendly bioremediation and biotransformation technologies. Deeper insight into the copper dependent regulation of sMMO also broadens our knowledge of bacteria dealing with high amount of heavy metal ions.

To date approximately 400 bacterial genomes are sequenced or under sequencing. Half of the sequenced genes are conserved but has unknown function. The transposon mutagenesis with the known genome sequence are good tools to undercover the biological function of unknown genes.

- Detailed examination of *mmoX* promoter region with bioinformatic tools
- Identification of minimal promoter region of *mmoX* with promoter probe vectors
- Sequencing and *in silico* analysis of DNA regions flanking *smmo* operon
- Mutagenesis of identified genes, phenotypic characterisation of mutant strains according to copper dependent expression of sMMO
- Optimisation of conjugation based gene delivery system for *M. capsulatus* (Bath)
- Development of transposon based mutagenesis method for *M. capsulatus* (Bath)
- Screening and identification of sMMO deficient mutants in transposon generated mutant library

METHODS

In vitro DNA and RNA manipulation analysis was done according to the general practice or the specification of manufacturers. To introduce recombinant DNA into *M. capsulatus* (Bath) a conjugation based gene transfer system was optimised and applied. Site directed and transposon mutagenesis was developed and employed to create mutants. sMMO enzyme assay was performed on various mutants to determine enzyme activity. Western hybridisation method was used to detect sMMO subunits. Green fluorescent protein as reporter was used in promoter probing vectors to determine promoter activity of various DNA fragments. Web based and local bioinformatic tools were used to analyse DNA and amino acid sequence data.

RESULTS

I. I identified σ^N promoter like DNA sequence motives in the 5' region of *mmoX*: the σ^N and the IHF (Integration Host Factor) binding site.

II. I demonstrated that the DNA region between 358-280 5' direction from *mmoX* ATG codon, just upstream from IHF binding site is indispensable for copper dependent sMMO expression.

III. I determined the DNA sequence of a 9.5 kb long region downstream from *mmoC* (3' direction). In this region I identified *mmoG*, *mmoQ*, *mmoS* and *mmoR* genes.

IV. I showed that in the *mmoXYBZCG* operon, containing the structural genes of sMMO, *mmoXY* and *mmoCG* intergenic regions have no promoter activity. It was suggested that all the structural genes: *mmoXYBZCG* are transcribed from the σ^N promoter in the 5' region of *mmoX*.

V. I identified σ^{70} like promoter motives in the 5' regions of *mmoS* and *mmoR*.

VI. With bioinformatic tools I predicted the function of *mmoG*, *mmoQ*, *mmoS* and *mmoR* genes based on amino acid sequence similarities: putative MmoG is a GroEL like chaperonin, MmoS domain structure is very similar to the sensor protein of the two-component sensor-regulator systems. MmoQ is like the regulator of two-component systems. MmoR domain structure identical to the σ^N-dependent transcriptional activators.

VII. I designed and produced *M. capsulatus* (Bath) mutant strains that contains mutations in *mmoG*, *mmoQ*, *mmoS* and *mmoR* genes respectively. I examined the growth rate, sMMO activity, the presence of the *mmoX* mRNA and the presence of the α, β and γ subunits of sMMO under copper-free and copper containing medium. I showed that *mmoG* and *mmoR* are indispensable for sMMO expression under copper-free conditions.

VIII. I demonstrated that the promoter in the 5' region of *mmoX* is constitutive in Δ*mmoQ* and Δ*mmoS* mutant strains. In 2μM copper containing medium Δ*mmoQ* and Δ*mmoS* strains showed sMMO activity but the wild type strain had no sMMO activity at all.

IX. I showed that in 5μM containing medium Δ*mmoQ* and Δ*mmoS* did not express active sMMO. I demonstrated that although *mmoX* constitutively transcribed when the medium contained 5μM copper-sulphate, there were no detectable sMMO subunits. I suggested that copper inactivated sMMO molecules degraded quickly under these conditions.

X. I developed a highly efficient transposon based mutagenesis system for *M. capsulatus* (Bath). This permits the positive selection of mutants in which single, random insertion has occured. The transposon also tags the mutated genes, thus the identification and isolation of the corresponding genomic region is simple. This is the first report on high frequency transposon mutagenesis in *M. capsulatus* (Bath), and should be useful to study various biological processes of *M. capsulatus* (Bath) and uncover the function of uncharacterized genes.

XI. I produced a transposon mutant library of *M. capsulatus* (Bath) and developed a screening method to detect sMMO deficient mutants. I isolated 77 sMMO deficient mutants. I determined

the site of integration of the transposon in 5 mutant strains. Based on the complete genome sequence of *M. capsulatus* (Bath) I determined the flanking genes of the mutated gene. Since the mutated genes and operons are quite diverse I suggested that the copper dependent regulation of SMMO is a complex process in *M. capsulatus* (Bath).

PUBLIKÁCIÓK

R. Csáki, L. Bodrossy, J. Klem, J. C. Murrell & K. L. Kovacs. Genes involved in the copper-dependent regulation of soluble methane monooxygenase of Methylococcus capsulatus (Bath): cloning, sequencing and mutational analysis Microbiology (2003) 149: 1785-1795

R. Csáki. Investigation of the copper-regulated expression of methane monooxygenases in Methylococcus capsulatus (Bath). Acta Biologica Szegediensis (2002) 46 (1-2):31

Fodor BD, Kovacs AT, **Csáki R**, Hunyadi Gulyas E, Klement E, Maroti G, Meszaros LS, Medzihradszky KF, Rakhely G, Kovacs KL. Modular broad-host-range expression vectors for single protein and protein complex purification. APPL ENVIRON MICROB 70: (2) 712-721 (2004)

Bodrossy L., T.Hanczár, **R.Csáki** és K.L.Kovács. 1999. Metánfaló baktériumok. Élet és Tudomány. 40, 1254-1256.

Csáki R., Hanczár T., Klem J., Panmerr A. és Kovács L. K. A metán anyagcsere réz-függő szabályozása metanotrófokban. Lecture presented at Straub Days, MTA SzBK, Szeged, Hungary, 25-30 Nov 2001.

Csáki R., T.Hanczár, L.Bodrossy, J.C.Murrell és K.L.Kovács. 2001. Molecular characterization of structural genes coding for a membrane bound hydrogenase in *Methylococcus capsulatus* (Bath). FEMS Microbiol Lett. 205, 203-207.

T.Hanczár, **R.Csáki**, L.Bodrossy, J.C.Murrell és K.L.Kovács. 2002. Detection and localisation of two hydrogenases in *Methylococcus capsulatus* (Bath) and their potential role in CH_4 metabolism. Archives of Microbiology. 177, 167-172.

K. L. Kovács, Cs. Bagyinka, L. Bodrossy, **R. Csáki**, B. Fodor, K. Györfi, T. Hanczár, M. Kálmán, J. Ősz, K. Perei, B. Polyák, G. Rákhely, M. Takács, A. Tóth, J. Tusz, Recent advances in biohidrogen research. Pflügers Arch – Eur. J. Physol. (2000) 439:81-83.

Kovács K.l, Bagi Z., Bagyinka Cs., Bodrossy L., **Csáki R.**, Fodor B., Hanczár T., Jennifer T., Kálmán M., Klem J., Kovács Á., Jian L., Magony M., Maróti G., Perei K., Polyák B., Solmaz A., Takács M., Tóth A., Rákhely G.. Biohidrogén, Biogáz, Bioremediáció. Acta Biol. Debrecina (2000) Vol. 22

Bodrossy L., I.R.McDonald, T.Hanczár, **R.Csáki**, G.Rákhely, J.C.Murrell, K.L.Kovács, Approaches to broaden the biotechnological potential of thermophilic methanotrophs. Poster presented at the Gordon Conference on the Molecular Basis of Microbial One-Carbon Metabolism, Henniker, New Hampshire, USA, 28 June - 3 July 1998.

T.Hanczár**, R.Csáki**, L.Bodrossy, J.C.Murrell, K.L.Kovács, Hydrogen metabolism in *Methylococcus capsulatus* (Bath). Poster presented at the 143rd SGM Meeting, Edinburgh, UK, 12-16 April 1999.

T.Hanczár**, R.Csáki**, L.Bodrossy, J.C.Murrell, K.L.Kovács, Hydrogenases in methanotrophic bacteria. Poster presented at the 13th International Congress of the Hungarian Society for Microbiology, Budapest, Hungary, 30th August – 1st September 1999.

T.Hanczár**, R.Csáki**, L.Bodrossy, J.C.Murrell, K.L.Kovács, Hydrogen driven methane monooxygenase activities in *Methylococcus capsulatus* (Bath). Poster presented at the 6th International Conference on the Molecular Biology of Hydrogenases, Berlin, Germany, 5th -10th August 2000.

R.Csáki., L.Bodrossy, T.Hanczár, J.Klem and K.L.Kovács, Improvement and application of molecular biological techniques for the investigation of *Methylococcus capsulatus* (Bath).
Poster presented at the Gordon research Conference on Molecular Basis of Microbial One Carbon Metabolism, Connecticut College, New London, USA, 8-13 July 2000.

T.Hanczár**.**, **R.Csáki**, L.Bodrossy, J.Klem, C.J.Murrell, Kornél L. Kovács, Hydrogenases in methanotrophic bacteria. Lecture presented at COST 841 action and IEA Annex 15 Joint Workshop, Szeged, Hungary, 7-12 Sept., 2001.

T.Hanczár, **Csáki R**., Bodrossy L. , Klem J., C.J. Murrell, Kovács L. K., Hidrogenázok metánfalókban, Lecture presented at Straub Days, MTA SzBK, Szeged, Hungary, 25-30 Nov. 2001.

T.Hanczár**, R.Csáki**, J.Klem és K.L.Kovács, Detection and localisation of two hydrogenases in *Methylococcus capsulatus* (Bath). Poster presented at the Gordon research Conference on Molecular Basis of Microbial One Carbon Metabolism, Connecticut College, New London, USA, 5-12. July 2002.

T.Hanczár, **R.Csáki**, J.Klem, L.Bodrossy, J.C.Murrell, and K.L. Kovács, Molecular and biochemical studies of soluble methane monooxygenase and hydrogenases in *Methylococcus capsulatus* (Bath).
Lecture presented at Hungarian Society for Microbiology Meeting, Balatonfüred, Hungary, 7-10 Oct. 2002 (in hungarian).