FINAL REPORT

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SUMMARY

At the start of this project, it was known that methanogens were *Archaeabacteria* (now *Archaea*) and were therefore predicted to have gene expression and regulatory systems different from Bacteria, but few of the molecular biology details were established. The goals were then to establish the structures and organizations of genes in methanogens, and to develop the genetic technologies needed to investigate and dissect methanogen gene expression and regulation *in vivo*. By cloning and sequencing, we established the gene and operon structures of all of the "methane" genes that encode the enzymes that catalyze methane biosynthesis from carbon dioxide and hydrogen. This work identified unique sequences in the methane gene that we designated *mcrA*, that encodes the largest subunit of methyl-coenzyme M reductase, that could be used to identify methanogen DNA and establish methanogen phylogenetic relationships. *McrA* sequences are now the accepted standard and used extensively as hybridization probes to identify and quantify methanogens in environmental research. With the methane genes in hand, we used northern blot and then later whole-genome microarray hybridization analyses to establish how growth phase and substrate availability regulated methane gene expression in *Methanobacterium thermautotrophicus* ΔH (now *Methanothermobacter thermautotrophicus*). Isoenzymes or pairs of functionally equivalent enzymes catalyze several steps in the hydrogen-dependent reduction of carbon dioxide to methane. We established that hydrogen availability determine which of these pairs of methane genes is expressed and therefore which of the alternative enzymes is employed to catalyze methane biosynthesis under different environmental conditions. As were unable to establish a reliable genetic system for *M. thermautotrophicus,* we developed *in vitro* transcription as an alternative system to investigate methanogen gene expression and regulation. This led to the discovery that an archaeal protein, designated TFE, that had sequences in common with the eukaryotic general transcription factor TFIIE, stimulated archaeal transcription initiation and that the archaeal TATA-box binding protein (TBP) remained attached to the promoter region whereas the transcription factor TFB dissociated from the template DNA following initiation. DNA sequences that directed the localized assembly of archaeal histones into archaeal nucleosomes were identified, and we established that transcription by an archaeal RNA polymerase was slowed but not blocked by archaeal nucleosomes. We developed a new protocol to purify archaeal RNA polymerases and with this enzyme and additional improvements to the *in vitro*

transcription system, we established the template requirements for archaeal transcription termination, investigated the activities of proteins predicted to be methane gene regulators, and established how TrpY, a novel archaeal regulator of expression of the tryptophan biosynthetic operon functions in *M. thermautotrophicus*. This also resulted in the discovery that almost all *M. thermautotrophicus* mutants isolated as spontaneously resistant to 5-methyl tryptophan ($5MT^R$) had mutations in *trpY* and were therefore $5MT^R$ through de-repressed *trp* operon expression. This established a very simple, practical procedure to determine and quantify the DNA sequence changes that result from exposure of this *Archaeon* to any experimental mutagenesis protocol. Following the discovery that the *Thermococcus kodakaraensis* was amenable to genetic manipulation, we established this technology at OSU and subsequently added plasmid expression, a reporter system and additional genetic selections to the *T. kodakaraensis* genetic toolbox. We established that transcription and translation are coupled in this *Archaeon*, and by combining *in vitro* transcription and *in vivo* genetics, we documented that both TFB1 and TFB2 support transcription initiation in *T. kodakaraensis.* We quantified the roles of ribosome binding sequences and alternative initiation codons in translation initiation, established that polarity exists in archaeal operon expression, and that oligoT-rich sequences terminate archaeal transcription *in vitro* and *in vivo*. Results of research supported by this grant have been reported in 41 primary peer-reviewed publications, and in 30 invited reviews and symposium contributions.

DETAILS

Plating, development of selectable genetic markers and replicons, and assays of *M. thermautotrophicus* transformation. Transformation requires DNA uptake, replication and expression in the recipient microbial cell, and we worked extensively on all three of these steps to establish transformation of *M. thermautotrophicus*. We established plating *M. thermautotrophicus* and obtained colony growth reproducibly with ~100% efficiency, on an agar-solidified salts medium in plates incubated at 65ºC in sealed containers containing a CO_2 plus H_2 atmosphere pressurized to 15 psi. We determined kill-curves and established procedures that resulted in the efficient recovery of *M. thermautotrophicus* cells following electroporation, exposure to many different osmotic and temperature shock regimes, and after protoplasting by exposure to a psuedomurein-digesting enzyme purified from *Methanobacterium wolfei*. Transformation of other methanogens was successful using a *Streptomycete*-derived puromycin-resistance conferring *pac* gene that provided an antibiotic selection for transformants. We used this gene extensively to evaluate many different protocols for transformation of *M. thermautotrophicus*, but without consistent success. As the encoded puromycin N-acetyl transferase might not have been stable at 65ºC, we attempted to isolate *pac* gene mutants that encoded thermostable variants of this enzyme by transformation of *Bacillus stearothermophilus* and selection for puromycin resistant growth at 55ºC and 65ºC. We determined the sensitivity of *M. thermautotrophicus* to more than fifty additional antibiotics, and from this established that pseudomonic acid, bleomycin, fluconazole and myxin could be used to select *M. thermautotrophicus* transformants. For all of these compounds, a resistance-conferring gene was available and we confirmed that they were

stable when incubated at 65ºC for several days. We obtained, and sequenced, several cryptic plasmids and phages from related thermophilic methanogens as potential sources of replicons, and also cloned several origins of replication from yeast. These replicons were cloned into established bacterial plasmids to construct shuttle vectors that would replicate and express genes in the cytoplasm of both *M. thermautotrophicus* and *Escherichia coli, Bacillus subtilis* or *S. cerevisiae*. We added strong archaeal promoters to ensure transcription of the selectable marker and replication genes, and optimized codon usage for gene expression in *M. thermautotrophicus*. Nevertheless, despite this effort, we were unable to establish consistent, reproducible transformation of *M. thermautotrophicus* with any combination of replicon, genetic selection and DNA uptake procedure.

Methane gene cloning, sequencing, and substrate regulation of their *in vivo* expression. The genes encoding the coenzyme F_{420}-dependent and F_{420}-independent hydrogenases and N^5,N^{10}-methenyltetrahydro-methanopterin reductases, coenzyme F_{420}-dependent N^5,N^{10}-methylenetetrahydro-methanopterin reductase, and an *fpa-orfX-rdxA* operon that encoded an abundant flavoprotein and a rubredoxin were cloned from *M. thermautotrophicus* and sequenced before the DOE sponsored sequencing of the entire *M. thermautotrophicus* genome was completed in 1997. With the genome sequence available, all the remaining recognizable *M. thermautotrophicus* methane genes were immediately cloned and incorporated into our expression studies. We discovered and characterized a very unusual protein that contained six sequential ferredoxin-like domains and was designated therefore a polyferredoxin. Several additional members of this family of intriguing family of presumably electron transport proteins have since been identified and remain a focus of investigations.

Northern blot analyses of RNA preparations isolated from *M. thermautotrophicus* cultures at different growth stages revealed that transcription of the *mth* gene that encodes one of the two enzymes that catalyze step 4 in the reductive pathway from CO_2 to CH_4 occurred during the earliest growth stages, presumably when the growth substrates (H_2 plus CO_2) were in excess, and was then replaced by transcription of the second (*mtd*) gene. Transcription of the *mer* gene (step 5 enzyme) followed the *mtd* pattern whereas transcription of the *mrtBDGA* operon that encodes on of the two step 7 catalyzing methyl coenzyme M reductases paralleled *mth* transcription. At later growth stages, transcription of the *mrt* operon was replaced by transcription of the *mcrBDCGA* operon that encodes the second step 7 catalyzing isoenzyme. *M. thermautotrophicus* cells were found to contain high levels of the *fpa*-encoded flavoprotein when grown under iron-limited conditions, and increased levels of the *mvhB*-encoded polyferredoxin when grown with excess iron.

Pairs of identical 2 l fermentors were used to grow experimental and control cultures concurrently to further investigate the observations of growth-phase-dependent methane gene expression. The changes in growth rate, methanogenesis, growth yield (Y_{CH4}) and methane gene expression were found to correlate with changes in the supply of dissolved H_2. With excess H_2 supply, biomass and methanogenesis increased exponentially and in parallel, and resulted in a constant Y_{CH4}, and transcription of the *mth*

gene and the *mrtBDGA* operon. Reducing the H_2 supply, by decreasing the percentage of H_2 in the input gas mixture or by decreasing the mixing speed of the fermentor impeller, decreased growth rate and resulted in a lower and constant rate of methanogenesis. Under H_2-limited growth conditions, cultures grew with a continuously increasing Y_{CH4} and the *mtd* gene and *mcr* operon were expressed. The changes in the kinetics of growth, methanogenesis and methane gene transcription that resulted from a reduction in the H_2 supply, were fully reversed by restoring a high H_2 supply. With the H_2 supply reduced to a level insufficient for growth, methanogenesis continued but at a low and constant rate, and only *mcr* expression occurred at a detectable level.

These investigations of the H_2-dependent regulation of methane gene transcription in *M. thermautotrophicus* were limited by the absolute dependence of this methanogen on H_2, under all conditions, for growth and energy generation. *Methanothermobacter thermoformicicum,* a very close relative of *M. thermautotrophicus*, however could grow and generate CH_4 under a N_2 atmosphere when provided with formate. The *fdhCAB* operon that encodes formate dehydrogenase was therefore cloned and sequenced from *M. thermoformicicum* and northern blot assays revealed that this operon was transcribed at all growth stages in *M. thermoformicicum* cells growing on formate, but also at very high levels at the late stages of growth of cultures of *M. thermoformicicum* on H_2 plus CO_2**.** Transcription of all of the other methane genes in *M. thermoformicicum* followed the patterns established for the homologous genes in *M .thermautotrophicus,* consistent with *fdh* expression also been regulated by the same environmental signals as *mtd, mer* and *mcr* transcription**.** Signals that result in relatively slow growth rates but high growth yields, an important feature of biological methanogenesis for environmental studies.

Using the *M. thermautotrophicus* genome sequence, microarrays were synthesized at TIGR and, in collaboration with Dr. Najib El-Sayed, microarray hybridization experiments established how substrate availability regulated gene expression on a genome-wide basis. In the first experiments, an impellor speed-change protocol was used to determine the transcripts present in RNA preparations isolated from cells growing with high H_2 (600 rpm fermentor mixing), and at increasing times after reducing the H_2 supply (200 rpm fermentor mixing), and then again after returning to a high H_2-supply (600 rpm mixing). The results were consistent with the northern blot expression data but, despite several repetitions and efforts to keep all experimental steps identical, control experiments revealed unacceptably different levels of the same transcript in RNA preparations from cells sampled at the same times after impellor shifts, but in different experiments. We concluded that the molecular events that followed a shift from high to low H_2 availability did not occur synchronously after the impellor speed change in all the cells in a *M. thermautotrophicus* population. We therefore changed the approach to identify all transcripts and compare their abundances in *M. thermautotrophicus* cells in balanced growth supplied with high or low H_2 in a turbidostat maintained at an OD_{600} of 0.2. Growth medium was pumped continuously into and out of the fermentor, and mixing held at 600 rpm or 200 rpm. A complete culture volume (1 l) was replaced between sampling, and multiple samples were collected sequentially from the turbidostat growing under each condition. Control hybridizations confirmed that there was very little variance in the levels of individual transcripts in repetitive RNA

samples isolated from cells growing with high or low H_2 supply. The results obtained established that 129 of the 1840 *M. thermautotrophicus* genes represented on the microarrays were transcribed at levels that differed >1.5-fold under the two H_2-supply conditions. Fifty-five genes were transcribed at higher levels under high H_2-conditions, of which seventeen encoded unknown or conserved hypothetical proteins and the remainder were methane genes or encoded proteins involved in glutamate metabolism, ion transport and gene expression. Seventy-four genes were transcribed at higher levels under low H_2 conditions, of which thirty-one encoded unknown or conserved hypothetical proteins and the remainder were methane genes or encoded proteins predicted to be involved in energy conservation, electron and uptake transport, cofactor metabolism and signaling. An additional pair of reciprocally-regulated, likely methane genes was identified, both of which encoded relatives of the β subunit of the coenzyme F_{420}-reducing hydrogenase. MTH0341 was transcribed under high H_2 and MTH0193 under low H_2 conditions.

Development of an *M. thermautotrophicus in vitro* transcription system. In the absence of genetics, an *in vitro* transcription system was needed to investigate the regulation of methane gene expression established *in vivo*. This was developed with the NATO-sponsored collaborative help of Prof. M. Thomm (Univ. Regensburg, Germany). Using DNA templates that carried a promoter that directed transcription of a protein (HMtB) or stable RNA ($tRNA^{val}$) encoding gene, a transcription system was established that contained purified native *M. thermautotrophicus* RNA polymerase (RNAP), recombinant TATA-binding protein (TBP) and transcription factor B (TFB). In this system, transcription initiation also occurred *in vitro* from the *M. thermautotrophicus fpaA*, *rpoN* and $tRNA^{gly}$ promoters, but not from the promoters of the *mrt, mcr*, *hmd, mtd* or *ftr* methane genes, the *trp* operon, or the TBP- and TFB-transcription factor encoding genes. Apparently additional factors, or modifications of the transcription system were needed to obtain transcription for these promoters *in vitro*, and considerable effort was invested in identifying and purifying these factors. Antibodies were raised against recombinant TBP and TFB and used to locate these transcription factors in larger complexes in fractions obtained from cell lysate by column chromatographic, sucrose and glycerol gradients. We identified an additional "missing" subunit of the archaeal RNAP, a discovery that facilitated subsequent RNAP structure-function studies, and demonstrated that TFB co-purified with the RNAP but did not identify any additional initiation-stimulating transcription factor. Bioinformatics predicted that a protein encoded in all archaeal genomes, designate TFE, was related to the α-subunit of the eukaryotic general transcription factor, TFIIE. When we added recombinant TFE to the *in vitro* transcription system, there was a modest (~2-fold) stimulation of initiation from some, but not all *M. thermautotrophicus* promoters. As transcription initiation occurred from only a limited number of promoters, and noticeably not from some promoters that were very active in vivo, we concluded that a new approach was needed to purify archaeal RNAPs. With RNAP preparations purified by the new protocol, initiation occurred in vitro from almost all *M. thermautotrophicus* (and later *T. kodakaraensis*) promoters *in vitro*, and template directed intrinsic transcription termination also occurred in vitro for the first time, and so could be further investigated.

Mechanics of archaeal transcription initiation, elongation and termination. The archaeal pre-initiation complex (PIC) was known to assemble with TBP bound to the TATA-box, TFB bound to both the BRE upstream of the TATA-box and to DNA ~25 bp downstream of the TBP/TATA complex, but the fates of TFB and TBP following initiation were unknown. We developed promoter competition and clearance assays that revealed that TFB was released soon after, but not coincident with initiation, whereas TBP remained attached to the template DNA. The *M. thermautotrophicus* genome encodes three archaeal histones (HMtA1, HMtA2 and HMtB) and transcription templates were constructed to which HMtA2 bound and assembled into an archaeal nucleosome at one precise location downstream from the site of transcription initiation. With this complex, we were able to determine how the presence of an archaeal nucleosome effected transcript elongation by the archaeal RNAP purified from *M. thermauto-trophicus*. Transcription was slowed from ~20 nt/sec to ~3 nt/sec when the RNAP encountered the archaeal nucleosome, and the patterns of transcripts generated revealed that the RNAP paused both before and within the archaeal nucleosome, but predominantly at the 5'-boundary of the nucleosome. In contrast to the continued progress of the elongating RNAP through an archaeal nucleosome, when the promoter was bound by an archaeal histone and assembled into an archaeal nucleosome, archaeal transcription initiation *in vitro* was completely inhibited.

In 2003, *T. kodakaraensis*, an euryarchaeon related to *M. thermautotrophicus*, was reported to be naturally competent for DNA uptake and amenable to genetic manipulation (Sato *et al*., J. Bacteriology 185:210-220). With this model system established at OSU, we were able to add *in vivo* approaches to our investigations of the requirements and the mechanics of archaeal transcription and gene expression. The genome sequence of *T. kodakaraensis* encodes two TFBs, designated TFB1 and TFB2, and it was predicted for many years that *Archaea* with more than one TFB employed the alternative factors to initiate transcription from different promoters. After establishing the technology to grow and genetically manipulate *T. kodakaraensis* at OSU, the first project pursued was to test this prediction. Using recombinant *T. kodakaraensis* TFB1, TFB2, TBP and native RNAP, we demonstrated that TFB1 and TFB2 were fully interchangeable. In contrast to predicted result, TFB1 and TFB2 did not exhibit promoter preferentiality, and as we were able to construct *T. kodakaraensis* strains with either TFB1 or TFB2 deleted, these factors were also equally able to support growth under laboratory conditions. In prokaryotes, with no nuclear membrane, ribosomes have the opportunity to bind and initiate translation before an mRNA is complete, and the close coupling of translation and transcription was well documented and exploited widely in *Bacteria* for gene regulation. The presence or absence of a closely associated ribosome determines if a transcribing bacterial RNAP terminates or continues to transcribe through a potential transcription terminator (attenuator) sequence. Similarly, premature translation termination by a stop codon increases downstream transcription termination, resulting in the phenomenon known as bacterial operon polarity. In collaborations, we confirmed by electron microscopy that translation and transcription are closely coupled in *T. kodakaraensis* and that polarity also exists in archaeal operon expression. We introduced a nonsense mutation into a promoter proximal gene and confirmed that this reduced the downstream expression of a β-glycosidase-encoding reporter gene (TK1761) in *T. kodakaraensis*.

This result predicted that archaeal transcription in *T. kodakaraensis* was sensitive to intrinsic termination by template sequences. This was confirmed by construction of strains in which putative archaeal transcription terminators were positioned between a strong archaeal promoter and the reporter gene. Expression of the reporter gene was almost completely inhibited by the presence upstream of oligoT-rich sequences. The ability of these sequences to terminate transcription by the archaeal RNAP purified from T. kodakaraensis was confirmed directly by using in vitro transcription assays. Expression of TK1761 was also used to confirm and quantify the activities *in vivo* of sequences predicted to be archaeal ribosome binding sites and alternative translation-initiating codons.

Promoter regulation. We generated DNA molecules that contained multiple copies of the promoter-containing intergenic regions from upstream of all of the methane genes established to be regulated by hydrogen availability in *M. thermautotrophicus*. These regulatory-region-carrying DNAs were attached to streptavidin-coated paramagnetic beads, and incubated with clarified cell extracts and with fractions obtained by column chromatographic separations of extracts of *M. thermautotrophicus* cells grown with low- or high-hydrogen availability. By mass spectrometry, we identified *M. thermautotrophicus* proteins that bound specifically to the regulator-DNA-coated beads and not to control beads. The genes encoding these proteins were cloned, and recombinant versions of these proteins were purified and added to *in vitro* transcription assays containing templates with the appropriate regulated methane gene promoter. We were never able to reproduce *in vitro* the hydrogen-dependent regulation of methane gene promoter function documented *in vivo*. To investigate if this regulation required more than one regulatory protein, and/or the presence of small effector molecules (possibly oxygen sensitive), aliquots of many different *M. thermautotrophicus* cell extracts and column fractions were added to *in vitro* transcription reactions mixtures that contained two template DNAs. One template had a methane gene promoter positioned to direct transcription of a 386 bp G-less sequence, and the second template had a control promoter positioned to direct transcription of a 196 bp G-less sequence. On several occasions we had an initial positive result, namely an increase in the ratio of the 386 nt versus 186 nt G-less transcript, but further purification steps never resulted in the isolation of a factor that stimulated or repressed initiation specifically from a methane gene promoter.

To add confidence that the *M. thermautotrophicus in vitro* transcription system established could be used to dissect promoter regulation, if supplied with all the necessary components, we undertook to reproduce *in vitro* the regulation of the *M. thermautotrophicus trpEGCFBAD* operon documented *in vivo*. The *in vivo* observations predicted that this operon was repressed in cells growing in the presence of tryptophan and a gene, designated *trpY*, located immediately upstream and transcribed divergently from the *trpEGCFBAD* operon was predicted to encode a transcription regulator. By using *in vitro* transcription, gel shift assays and DNase I footprinting with *M. thermautotrophicus* RNAP, TBP, TFB and TrpY, and DNA molecules with wild-type and mutated versions of promoter regions, we established that TrpY bound tryptophan and also bound to operator sequences (consensus 5'-GTGACA; designated TRP-boxes) located between *trpY* and the *trpEGCFBAD* operon. TrpY binding inhibited *trpY*

transcription, and therefore its own synthesis, in the absence of tryptophan. In the presence of tryptophan, TrpY binding also inhibited transcription of the *trpEGCFBAD* operon. TRP-box binding by TrpY was shown to block RNAP access to the site of *trpY* transcription initiation, and to prevent TBP and TFB binding to the BRE-TATA-box region of the *trpEGCFBAD* promoter. The *M. thermautotrophicus* genome encodes a second, unlinked *trpB* gene (*trpB2*). TrpY was shown to bind to TRP-box sequences upstream of *trpB2* in both the absence and presence of tryptophan, but only inhibited *trpB2* transcription in the presence of tryptophan. Bioinformatics predicted that TrpY had an N-terminal helix-turn-helix (HTH) DNA-binding motif and a C-terminal, presumably tryptophan, ligand-binding ACT-domain. We showed that TrpY bound ^{14}C-tryptophan and discovered that spontaneous resistance to 5-methyl tryptophan (5MT^{R}) resulted in *M. thermautotrophicus* from mutations that eliminated TrpY function. Over 100 *trpY* genes were PCR amplified and sequenced from 5-MT^{R} *M. thermautotrophicus* mutants. Most contained nonsense mutations but 26 different missense mutations were identified and recombinant versions of the encoded TrpY variants were generated. Consistent with the bioinformatics predictions, variants with amino acid substitutions in the N-terminal HTH region no longer bound DNA but did still bind tryptophan, whereas variants with changes in the ACT domain did not bind tryptophan but still bound DNA. Surprisingly, some variants that did not inhibit *trpY* or *trpEGCFBAD* transcription *in vivo*, still bound TRP-box DNA and ^{14}C-tryptophan *in vitro*. Micrococcal footprinting studies revealed that an additional event, TrpY oligomerization, was required for TrpY binding to inhibit *trp* gene transcription *in vitro*. Sequence-specific binding followed by oligomerization of the regulator to form a complex that incorporates the promoter region has since been reported for several additional archaeal transcription regulators. The discovery that almost all 5-MT^{R} mutants of *M thermautotrophicus* have sequence changes in *trpY* has provided a very simple method to identify and quantify mutations that occur in an *Archaeon* following exposure to any experimental mutagenesis regime.

PUBLICATIONS

Primary Research Reports documenting results of research supported by this award:

1. Thomm, M., Sherf, B.A. and Reeve, J.N. 1988. The RNA polymerase binding and transcription initiation sites upstream of the methyl reductase operon in *Methanococcus vannielii*. J. Bacteriol. *170*:1958-1961.

2. Morris, C.J. and Reeve, J.N. 1988. Conservation of structure in the human gene encoding argininosuccinate synthetase and the *argG* genes of the archaebacteria *Methanosarcina barkeri* and *Methanococcus vannielii*. J. Bacteriol. *170*:3125-3130.

3. Weil, C.F., Cram, D.S., Sherf, B.A. and Reeve, J.N. 1988. Structure and comparative analysis of the genes encoding component C of methyl coenzyme M reductase in the extremely thermophilic archaebacterium *Methanothermus fervidus*. J. Bacteriol. *170*:4718-4726.

4. Reeve, J.N., Beckler, G.S., Cram, D.S., Hamilton, P.T., Brown, J.W., Krzycki, J.A., Kolodziej, A, Alex, L., Orme-Johnson, W.H. and Walsh, C.T. 1989. A hydrogenase gene in *Methanobacterium thermoautotrophicum* encodes a polyferredoxin. Proc. Natl. Acad. Sci. USA *86*:3031-3035.

5. Lunnen, K.D., Morgan, R., Timan, C.J., Krzycki, J.A., Reeve, J.N. and Wilson, G.G. 1989. Characterization and cloning of *MwoI* (GCN_5/N_2GC), a new restriction enzyme from the thermophilic archaebacterium *Methanobacterium wolfei*. Gene *77*:11-19.

6. Brown, J.W. and Reeve, J.N. 1989. Transcription initiation and a RNA polymerase binding site upstream of the *purE* gene of the archaebacterium *Methanobacterium thermoautotrophicum* strain H. FEMS Microbiol. Lett. *60*:131-136.

7. Haas, E.S., Brown, J.W., Daniels, C.J. and Reeve, J.N. 1990. Genes encoding the 7S RNA and a $tRNA^{Ser}$ are linked to one of the two rRNA operons in the genome of the extremely thermophilic archaebacterium *Methanothermus fervidus*. Gene *90*:51-59.

8. Sherf, B.A. and Reeve, J.N. 1990. Identification of gp*mcrD* and its association with component C of methyl coenzyme M reductase in *Methanococcus vannielii*. J. Bacteriol. *172*:1828-1833.

9. Alex, L., Reeve, J.N., Orme-Johnson, W.H. and Walsh, C.T. 1990. Cloning, sequence determination and expression of the genes encoding the subunits of the Ni-containing 8-hydroxy-5-deazaflavin reducing hydrogenase from *Methanobacterium thermoautotrophicum* H. Biochem. *29*:7237-7244.

10. Steigerwald, V.J., Beckler, G.S. and Reeve, J.N. 1990. Conservation of hydrogenase and polyferredoxin structure in the hyperthermophilic archaebacterium *Methanothermus fervidus*. J. Bacteriol. *172*:4715-4718.

11. Steigerwald, V.J., Pihl, T.D. and Reeve, J.N. 1992. Identification and isolation of the polyferredoxin from *M. thermoautotrophicum* H strain Proc. Natl. Acad. Sci. USA *89*:6929-6933.

12. Stroup, D., and Reeve, J.N. 1993. Association of the *mcr*D gene product with methyl coenzyme M reductase in *Methanococcus vannielii*. Biochim. Biophys. Acta. *1203*:175-183.

13. Stroup, D. and Reeve, J.N. 1993. Identification of the *mcr*C gene product in *Methanococcus vannielii*. FEMS. Microbiol. Lett. *111*:129-134.

14. Hennigan, A.N. and Reeve, J.N. 1994. mRNAs in the methanogenic archaeon *Methanococcus vannielii*: numbers, half-lives and processing. Mol. Microbiol. *11*:655-670.

15. Pihl, T.D., S. Sharma and Reeve, J.N. 1994. Growth phase dependent transcription of the genes that encode the two methyl coenzyme M reductase isoenzymes and N^5-methyltetrahydromethanopterin: coenzyme M methyltransferase in *Methanobacterium thermoautotrophicum* H strain J. Bacteriol. *176*:6384-6391.

16. Nölling, J., Pihl, T.D., Vriesema, A. and Reeve, J.N. 1995. Organization and growth phase dependent transcription of methane genes in two regions of the *Methanobacterium thermoautotrophicum* genome. J. Bacteriol. *177*:2460-2468.

17. Nölling, J., Ishii, M., Koch, J., Pihl, T.D., Reeve, J.N., Thauer, R.K. and Hedderich, R. 1995. Characterization of a 45 kDa flavoprotein and evidence for a rubredoxin, two proteins that could

participate in electron transport from H_2 to CO_2 in methanogenesis in *Methanobacterium thermoautotrophicum*. Eur. J. Biochem. *231*:628-638.

18. Kim, B-K., Pihl, T.D., Reeve, J.N. and Daniels, L. 1995. Purification of the copper-response extracellular proteins secreted by the copper-resistant methanogen *Methanobacterium bryantii* strain BKYH, and cloning, sequencing and transcription of the gene encoding these proteins. J. Bacteriol. *177*:7178-7185.

19. Nölling, J., Pihl, T.D. and Reeve, J.N. 1995. Cloning, sequencing and growth phase-dependent transcription of the coenzyme F_{420}-dependent N^5,N^{10}-methylenetetrahydro-methanopterin reductase encoding genes from *Methanobacterium thermoautotrophicum* ΔH and strain *Methanopyrus kandleri*. J. Bacteriol. *177*:7238-7244.

20. Nölling, J., Elfner, A., Palmer, J.R., Steigerwald, V.J., Pihl, T.D., Lake, J.A. and Reeve, J.N. 1996. The phylogeny of *Methanopyrus kandleri* and methyl coenzyme M reductase operons. Int. J. System. Bacteriol. *46*:1170-1173.

21. Morgan, R.M., Pihl, T.D., Nölling, J., and Reeve, J.N. 1997. Hydrogen regulation of growth, growth yields and methane gene transcription in *Methanobacterium thermoautotrophicum* ΔH. J. Bacteriol *179*:889-898.

22. Nölling, J., and Reeve, J.N. 1997. Growth and substrate dependent transcription of the formate dehydrogenase (*fdhCAB*) operon in *Methanobacterium thermoformicicum* strain Z-245. J. Bacteriol. *179*:899-908.

23. Pereira, S.L., and Reeve, J.N. 1999. Archaeal nucleosome positioning sequence from *Methanothermus fervidus*. J. Mol. Biol. *289*:675-681.

24. Darcy, T.J., Hausner, W., Awery, D.E., Edwards, A., Thomm, M., and Reeve, J.N. 1999. *Methanobacterium thermoautotrophicum* RNA polymerase and transcription *in vitro*. J. Bacteriol. *181*:4424-4429.

25. Li, W-T., Sandman, K., Pereira, S.L., and Reeve, J.N. 2000. MJ1647 encodes a very thermostable archaeal histone with a C-terminal extension in the hyperthermophile *Methanococcus jannaschii*. Extremophiles *4*: 43-51.

26. Wasserfallen, A., Nölling, J., Pfister, P., Reeve, J. and Conway de Macario, E. 2000. Phylogenetic analysis of 18 thermophilic *Methanobacterium* isolates supports the proposals to create a new genus, *Methanothermobacter* gen. nov., and to reclassify all isolates in three species, *Methanothermobacter thermoautotrophicus* comb. nov., *Methanothermobacter wolfeii* comb. nov., and *Methanothermobacter marburgensis* sp. nov. Int. J. System. Evol. Microbiol. *50*: 43-53.

27. Soares, D.J., Sandman, K., and Reeve, J.N. 2000. Mutational analysis of archaeal histone-DNA interactions. J. Mol. Biol. *297*: 39-47.

28. Bailey, K. A., Pereira, S.L., Widom, J., and Reeve, J.N. 2000. Archaeal histone selection of nucleosome positioning sequences and the procaryotic origin of histone-dependent genome evolution. J. Mol. Biol. *303*: 25-34.

29. Hanzelka, B., Darcy, T.J. and Reeve, J.N. 2001. TFE, an archaeal transcription factor in *Methanobacterium thermoautotrophicum* that is related to eucaryal transcription factor TFIIE J. Bacteriol. *183*:1813-1818.

30. Xie, Y. and Reeve, J.N. 2004. Transcription by an archaeal RNA polymerase is slowed but not blocked by an archaeal nucleosome. J. Bacteriol. *186*:3492-3498.

31. Xie, Y. and Reeve, J.N. 2004. Transcription by *Methanothermobacter thermautotrophicus* RNA polymerase *in vitro* releases archaeal transcription factor B but not TATA-box binding protein from the template DNA. J. Bacteriol. *186*:6303-6310.

32. Xie, Y. and Reeve, J.N. 2005. Regulation of tryptophan operon expression in the Archaeon *Methanothermobacter thermautotrophicus.* J. Bacteriol. *187*:6419-6429.

33. Santangelo, T.J. and Reeve, J.N. 2006. Archaeal RNA polymerase is sensitive to intrinsic termination directed by transcribed and remote sequences. J Mol. Biol. *355*: 196-210.

34. Shin, J-H., Santangelo, T.J., Xie, Y., Reeve, J.N., and Kelman, Z. 2007. Archaeal MCM helicase can unwind DNA bound by archaeal histones and transcription factors. J. Biol Chem. *282*:4908-4915.

35. Santangelo, T.J., Cubonova, L., James, C.L., and Reeve, J.N. 2007. TFB1 or TFB2 is sufficient for *Thermococcus kodakarensis* viability and basal transcription *in vitro*. J. Mol. Biol. *367*:344-357.

36. French, S.L, Santangelo, T.J, Beyer, A.L., and Reeve, J.N. 2007. Transcription and translation are coupled in *Archaea*. Mol. Biol. Evol. *24*:893-895.

37. Cubonova, L, Sandman, K., Karr, E.A., Cochran, A.J., and Reeve, J.N. 2007. Spontaneous mutagenesis of *trpY* and mutational analysis of the TrpY archaeal transcription regulator. J. Bacteriol. *189*: 4338-4342

38. Santangelo**,** T.J., Cubonova, L., Masumi, R., Atomi, H., Imanaka, T., and Reeve, J.N. 2008 Polarity in archaeal operon transcription in *Thermococcus kodakaraensis*. J. Bacteriol. *190*:2244-2248.

39. Karr, E.A., Sandman, K., Lurz, R., and Reeve, J.N. 2008. TrpY regulation of trpB transcription in *Methanothermobacter thermautotrophicus*. J. Bacteriol. *190*:2379-2387.

40. Santangelo, T.J., Cubonova, L., and Reeve, J.N. 2008. Shuttle vector expression in *Thermococcus kodakaraensis:* contributions of *cis* elements to protein synthesis in a hyperthermophilic Archaeon. Appl. Environ. Microbiol. *74*:3099-3104.

41. Weidenbach, K., Glöer, J., Ehlers, C., Sandman, K., Reeve, J.N., and Schmitz, R.A. 2008. Deletion of the archaeal histone in *Methanosarcina mazei* Gö1 results in reduced growth and genomic transcription. Mol. Microbiol. *67*:662-671.

42. Hirata, A., Kanai, T., Santangelo, T.J., Tajiri, M., Manabe, K., Reeve, J.N., Imanaka, T., and Murakami, K.S. 2008. Archaeal RNA polymerase subunits E and F are not required for

transcription *in vitro*, but a *Thermococcus kodakarensis* mutant lacking subunit F is temperature-sensitive. Mol. Microbiol. *70*:623-633.

43. Louvel, H., Kanai, T., Atomi, H., and Reeve, J.N. 2009. The Fur iron regulator-like protein is cryptic in the hyperthermophilic archaeon *Thermococcus kodakaraensis*. FEMS Microbiol. Lett. *295*:117-128.

44. Santangelo, T.J., Cubonova, L., Skinner, K.M., and Reeve, J.N. 2009. Archaeal intrinsic transcription termination *in vivo*. J. Bacteriol. *191*: In Press

Reviews and symposium contributions that were invited based on the results obtained, and include results generated through this support

1. Reeve, J.N. 1988. Methane Genes. In *Anaerobes Today*. Ed. Hardie, J.E., J. Wiley and Sons. pp. 95-104.

2. Weil, C.F., Sherf, B.A. and Reeve, J.N. 1989. Comparison of methyl reductase genes and gene production of methanogenic archaebacteria. Canad. J. Microbiol. *35*:101-108.

3. Brown, J.W., Daniels, C.J. and Reeve J.N. 1989. Structure, organization and expression of archaebacterial genes. C.R.C. Critical Reviews in Microbiology *16*:287-338.

4. Reeve, J.N. 1989. Molecular Biology of Methanogens and Methanogenesis. In *Genetics and Molecular Biology of Industrial Microorganisms,* eds. Hershberger, C.L., Queener, S.W. and Hegeman, G. ASM Publications, Washington, D.C. 20006. pp. 207-214.

5. Reeve, J.N. and Beckler, G.S. 1990. Conservation of primary structure in procaryotic hydrogenases. FEMS Microbiol. Rev. *87*:419-424.

6. Reeve, J.N. and Sherf, B.A. 1990. Methanogenesis and methane genes. In: *Biotechnology for Energy*, edit. Malik, K.A., Mujtaba Naqvi, S.H. and Aleem, M.I.H., NIAB/NIBGE, Faisalabad, Pakistan pp. 91-100.

7. Palmer, J.R. and Reeve, J.N. 1991. Methanogen genes and the molecular biology of methane biosynthesis. In The Genetics and Molecular Biology of Anaerobes. ed. Sebald M. Springer-Verlag, New York, NY. pp. 13-35.

8. Reeve, J.N. 1992. Molecular biology of methanogens. Ann. Rev. Microbiol. *46*:165-191.

9. Palmer, J.R. and Reeve, J.N. 1993. Structure and function of methanogen genes. In: *The Biochemistry of Archaea (Archaebacteria)* ed. Kates M., Kushner, D.J. and Matheson A.T., Elsevier. Amsterdam New Compreh. Biochem. *26*:497-534.

10. Reeve, J.N. 1993. Structure and organization of genes. In: *Methanogenesis.* ed. Ferry, J.G. Chapman and Hall, New York. pp. 461-495.

11. Steigerwald, V.J., Stroup, D., Hennigan, A.H., Pihl, T.D., and Reeve, J.N. 1993. Genes encoding the methyl-viologen reducing hydrogenase, polyferredoxin and methyl coenzyme M reductase II are adjacent in the genomes of *Methanobacterium thermoautotrophicum* and

Methanothermus fervidus. In: *Microbial Growth on C1 Compounds*. Intercept Ltd., Andover, U.K. pp. 181-191.

12. Steigerwald, V.J., Stroup, D., Hennigan, A.N., Palmer, J.P., Pihl, T.D., Daniels, C.J. and Reeve, J.N. 1993. Methyl coenzyme M reductase II genes and their close linkage to the methyl viologen-reducing hydrogenase-polyferredoxin operon in the genomes of *Methanobacterium thermoautotrophicum* and *Methanothermus fervidus*. In*: Industrial Microorganisms: Basic and Applied Molecular Genetics*. eds. Baltz, R.H., Hegeman, G.D. and Skatrud, P.L. ASM Press Washington, D.C. pp. 109-115.

13. Reeve, J.N., Morgan, R.M., and Nölling, J. 1997. Environmental and molecular regulation of methanogenesis. Water Sci. Technol. *36*: 1-6.

14. Reeve, J.N., Nölling, J., Morgan, R.M., and Smith, D.R. 1997. Methanogenesis: genes, genomes, and who's on first. J. Bacteriol. *179*: 5975-5986.

15. Pereira, S.L., and Reeve, J.N. 1998. Histones and nucleosomes in *Archaea* and *Eukarya*: a comparative analysis. Extremophiles *2*:141-148.

16. Reeve, J.N. 1999. Archaebacteria then....Archaeas now. J. Bacteriol. *181*:3613-3617.

17. Reeve, J.N**.** 2003. Archaeal chromatin and transcription. Mol. Microbiol. *48*: 587-598.

18. Xie, Y., and Reeve, J.N. 2003. In vitro transcription assays using components from *Methanothermobacter thermautotrophicus*. Meth. Enzymol. *370*:66-72.

19. Reeve, J.N., and Schmitz, R.A. 2005. Biology, biochemistry and the molecular machinery of Archaea. Curr. Opin Microbiol. *8*: 627-629.

20. Reeve, J.N., and Sandman, K. 2007. Chromatin and regulation. In *Archaea: Evolution, Physiology, and Molecular Biology***.** Eds Garrett, R., and Klenk, H-P. Blackwell Publ., Oxford UK. pp 147-158.

21. Samson, R., and Reeve, J.N. 2007. DNA binding proteins and chromatin. In *Archaea: Molecular and Cellular Biology*. Ed. Cavicchioli, R. ASM Press, Washington, DC. USA. pp. 110-119.