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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

A DNA binding protein, designated HMf, and DNA dependent RNA polymerase (RNAP) have been isolated and characterized from the extremely thermophilic methanogen Methanothermus fervidus. Binding of HMf to double-stranded DNA increases its thermal denaturation temperature by as much as 25°. The isolated RNAP is oxygen stable and has optimum activity at 60° in the presence of 350 mM K^+. Addition of HMf at low protein:DNA ratios appears to increase transcription in vitro by the M. fervidus RNAP. Genes encoding the subunits of methyl coenzyme M reductase, non-F_{420}-reducing hydrogenase, tRNAs and rRNAs have been cloned and sequenced from M. fervidus and are being used as templates for in vitro transcription by the M. fervidus RNAP. The M. fervidus mvhB gene appears to encode a polyferredoxin. The mvhB DNA sequence predicts that the encoded protein contains six tandemly-repeated bacterial ferredoxin domains. Keywords')

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Annual Report

Contract #:   N00014-86-K-0211

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         Methanothermus fervidus

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1. Project Goals

The goals of this project are to determine how the genome of Methanothermus fervidus is stabilized and how genes are expressed in this archaebacterium at temperatures in excess of 80°C. The approach is to isolate DNA-binding proteins and DNA-dependent RNA-polymerase and to analyze the activities of these proteins using an in vitro transcription system. Several genes which are expected to be highly expressed in vivo have now been cloned and characterized from M. fervidus and are being used as templates for in vitro transcription.

2. Accomplishments

a) Purification and characterization of HMf. We have found that approximately 1% of the total cellular protein of Methanothermus fervidus is a very small protein (apparent molecular weight of 4,000 by SDS-PAGE) which binds very tightly, but reversibly, to DNA. We have purified this protein, (designated HMf), to homogeneity and have begun to characterize its interactions with DNA and effects on transcription. Based on the amount of HMf needed to saturate DNA molecules in vitro we estimate that approximately 25% of the genome of M. fervidus should be bound by HMf in vivo. HMf has a very positive net charge, appears to be a tetramer under native conditions and from spectroscopic analyses probably does not contain phenylalanine or tyrosine residues.

b) Isolation of DNA-dependent RNA polymerase (RNAP). Previous attempts to purify RNAP from M. fervidus resulted in a very unstable, oxygen-sensitive enzyme [(1); M. Thomm; Personal Communication 1988] which had only limited in vitro activity using poly-d(A-T) as the template. We have investigated several modifications of the procedure we developed to purify RNAP from Methanobacterium thermoautotrophicum (2) and now have isolated preparations of RNAP from M. fervidus which are very stable. Polymerization activity, based on transcription of either poly-d(A-T) or double-stranded plasmid DNAs, remains stable for several weeks in 10% glycerol at 4°C in the presence of oxygen. This is a major advance, having a methanogen-derived RNAP activity which does not require anaerobic conditions has very much accelerated the rate at which the in vitro parameters of its activity can be investigated. We have intentionally limited the purification to steps which generate a DNA-dependent, [3H]dATP-polymerizing activity. We have not attempted to isolate a 'pure' enzyme because we do not wish to purify away "auxiliary" transcription factors at this stage. The enzyme preparations currently being used contain approximately 20 polypeptides when analyzed by SDS-PAGE. Template dependent polymerization of [3H]-dATP is optimum at 60°C in the presence of 350mM KCl.

c) Interactions of HMf and RNAP activity. A crucial issue for this project is how enzymes, such as RNAP, which require local denaturation of ds DNAs for their activities function in the presence of large amounts of a very tight binding protein such as HMf. We have therefore begun to investigate the effects of HMf on M. fervidus RNAP activity in vitro. The initial results are very exciting. We have found that in the presence of HMf, at low HMf:DNA ratios (approximately 1% of the saturating ratio), there is a three-fold stimulation of [3H]-dATP polymerization activity by RNAP at 56°C. Stimulation is only two-fold at lower temperatures and marginal, if at all, at

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temperatures above 75 C. There is no stimulation, but also no inhibition of M. fervidus RNAP activity, at saturating ratios of HMf to DNA. Determining how HMf stimulates RNAP activity in vitro must be a major research activity for the immediate future.

d) Cloning M. fervidus genes. The goals of this aspect of the project were to clone and sequence M. fervidus genes which, in themselves, are inherently interesting and which would provide a range of templates for use in the in vitro transcription studies. Genes encoding the enzymes which catalyze two key steps in methanogenesis, namely hydrogenase and methyl co-enzyme M reductase, and genes encoding stable RNAs, namely tRNAs and rRNAs have now been cloned and sequenced. We therefore now have DNA templates which should contain very strong promoters and which direct transcription of either protein-encoding genes and/or RNA-encoding genes.

i) Methyl co-enzyme M reductase (MR) operon, mcrBDCGA. A 6Kbp region of the M. fervidus genome, which encodes the α, β and γ subunits of MR plus three unknown genes (mcrD, mcrC and ORF258) has been cloned and sequenced. This work has been published (3) and the primary structures of the M. fervidus MR polypeptides have been compared, in detail, with the functionally-equivalent, ancestrally-homologous polypeptides from two mesophilic methanogens (Methanococcus vannielii, Methanosarcina barkeri) and a moderately thermophilic methanogen (Methanobacterium thermoautotrophicum) (4). It is clear that the MR enzymes from the two thermophilic methanogens are most similar having approximately 80% homologous amino-acid residues. Several very highly conserved oligonucleotide sequences have been identified, present in all the mcr operons, which are being evaluated as DNA probes to identify and quantify all methanogens in environmental samples.

The sites at which transcription initiates and terminates flanking the mcrBDCGA operon in M. fervidus, have been identified. A sequence upstream of mcrB which most likely functions as the promoter for this operon has been identified (3,4). This region of DNA has now been subcloned and is being used as a template in the transcription studies described above. An unusual feature of the M. fervidus mcr operon is that there is no inverted repeat sequence downstream of mcrA preceding the site of transcription termination as is found downstream of the mcrA gene in all the other mcr operons so far sequenced from methanogens. Transcription terminates in M. fervidus immediately following four tandemly arranged oligo-T sequences. This strongly resembles the transcription termination signals which have been identified in other thermophilic but non-methanogenic archaebacteria (5). Using double-stranded RNA (hairpin-loop) structures to direct transcription termination is apparently not practical for these organisms which grow at such very high temperatures and studying transcription termination in extreme thermophiles should therefore be very interesting.

ii) Methyl-viologen reducing hydrogenase operon, mvhDGAB Methanothermus fervidus generates energy by using H_2 to reduce CO_2 and produce CH_4. Hydrogenase activity is therefore essential for growth of this species, and as the concentration of molecular hydrogen in an aqueous environment at 80 C must be very low, investigating hydrogenase(s) in M. fervidus should be very valuable. There are two distinct hydrogenases in the moderate thermophile Methanobacterium thermoautotrophicum and we have used genes cloned from this species (6) as hybridization probes to isolate related
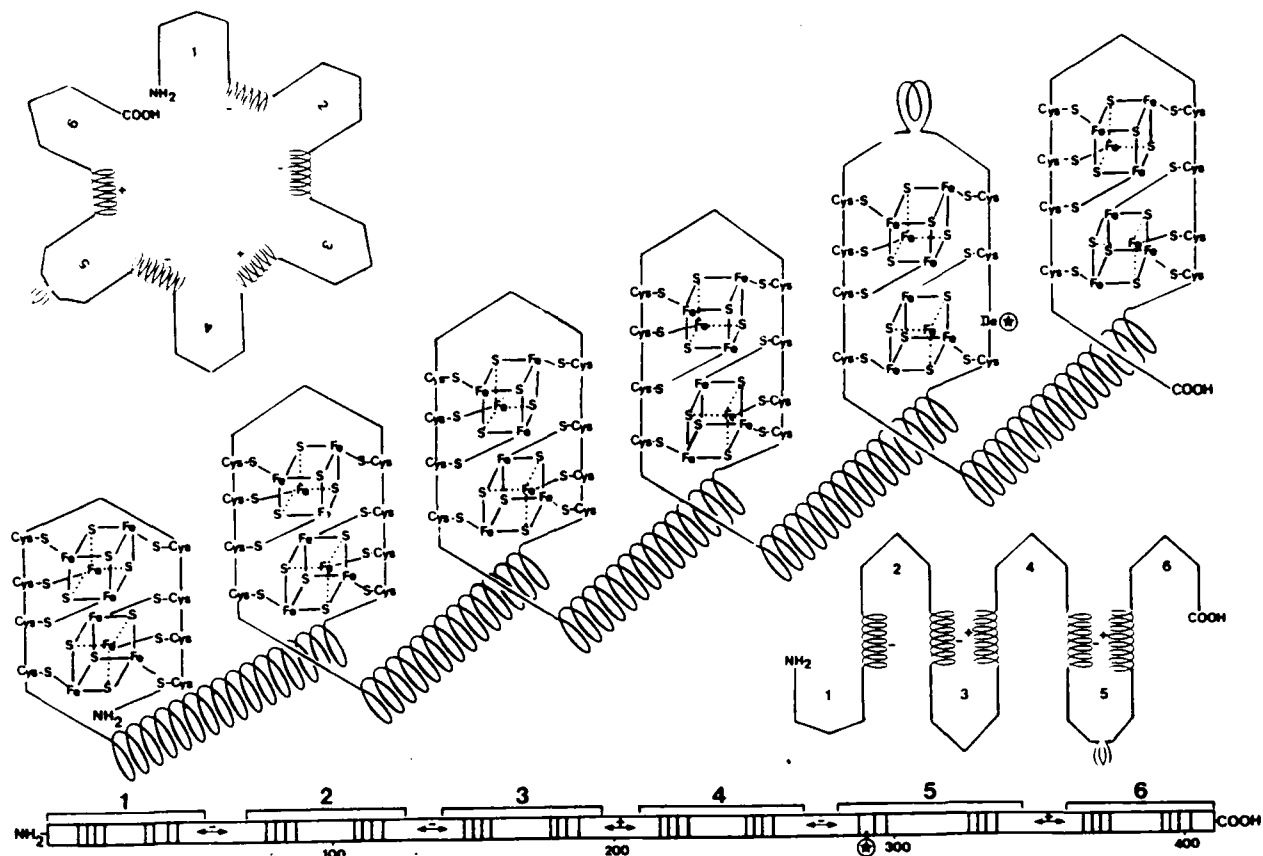
hydrogenase genes from M. fervidus. The results obtained, to date, indicate that M. fervidus has a cluster of four open reading frames which are ancestrally homologous to the mvhDGAB genes of M. thermoautotrophicum. These M. fervidus genes have been cloned, and sequencing is in progress. The most interesting gene is mvhB which apparently encodes a polymeric ferredoxin. In M. fervidus, as previously found in M. thermoautotrophicum (6), the mvhB encoded polypeptide should contain six tandemly-repeated bacterial ferredoxin domains. Figure 1 shows two of these domains with the differences between the M. thermoautotrophicum and M. fervidus proteins in these regions indicated. Determining structure-function relationships of this novel protein will be a major goal of our future research.

iii) Stable RNA encoding genes. The results we obtained by cloning and sequencing M. fervidus genes which encode two clusters of tRNAs and a 5SrRNA have now been published (7). They demonstrate that M. fervidus tRNAs contain slightly more base-paired regions than the functionally homologous tRNAs from mesophilic methanogens but that base-pairing alone cannot account for the increased thermal stabilities of M. fervidus tRNAs. The 5SrRNA sequence confirms that M. fervidus is closely related, phylogenetically, to M. thermoautotrophicum but also displays features not seen in the 5SrRNAs sequenced from other methanogens. The DNA regions upstream of the tRNA operons contain sequences which are virtually identical to the sequences identified as promoters for tRNA genes in M. vannielii. These are presumably the promoters for transcription of the M. fervidus tRNA genes and plasmids containing these sequences have been incorporated into the in vitro transcription studies.

We have found that the genome of M. fervidus contains two copies of the 16S-23S-5S ribosomal RNA operon. Both operons have been cloned and sequencing of one operon is in progress. This will provide additional data for phylogenetic studies, for evaluation in terms of the thermal stabilities of M. fervidus RNAs and will also provide additional promoter region(s) for use in transcription studies.

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(2) Brown, J.W. and Reeve, J.N. 1989. FEMS Microbiol. Letter. In Press.

(3) Weil, C.F., Cram, D.S., Sherf, B.A. & Reeve, J.N. 1988. J. Bacteriol. 170:4718-4726.

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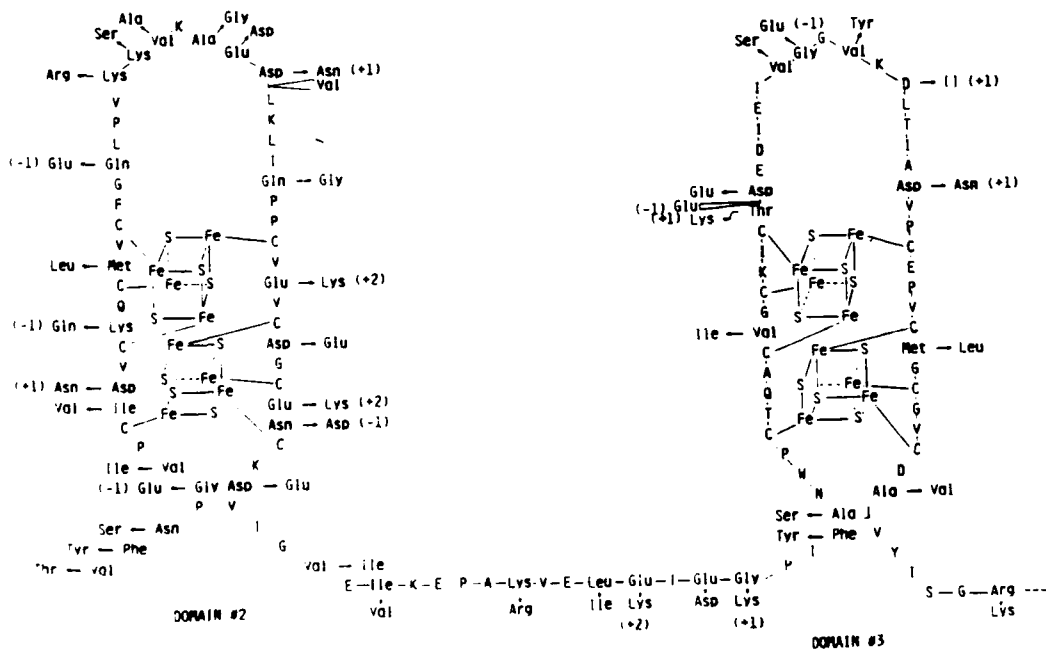
Figure 1. Structure of the polyferredoxin encoded by mvhB. The upper diagram shows the overall structure of the six-tandemly repeated bacterial ferredoxin domains with potential variations in secondary structure. The lower diagram compares the amino-acid sequences of domains 2 and 3 and the connector region from M. thermoautotrophicum (inner sequence) and M. fervidus (outer sequence). Charge changes are indicated as +/-.

(7) Haas, E.S., Daniels, C.J. & Reeve, J.N. 1989. Gene. _77_:253-263.

3. Objectives for Year 2

a) HMf. The primary structure of HMf will be determined both directly via microsequencing, and indirectly by cloning the encoding gene. This will allow a comparison with other DNA binding proteins, will enable us to express the HMf-encoding gene in E. coli and facilitate in vitro site-specific mutagenesis of the encoding gene. The in vitro interaction of HMf with DNA will be characterized by electron microscopy and antibodies raised against HMf will be used to localize HMf in vivo.

b) RNAP. We will continue to try variations of our RNAP purification scheme with the goal of obtaining an enzyme preparation which initiates transcription specifically and only at M. fervidus promoter sequences. We will use the promoter regions subcloned from the mcr, mvh and stable RNA operons as templates for development of the in vitro transcription system. Plasmids which contain identical sequences except for the M. fervidus promoter region(s) will provide negative controls.

c) HMf and RNAP interactions. The stimulation of M. fervidus RNAP activity by HMf will be pursued. Obvious questions are: is this limited to a specific preparation of M. fervidus RNAP? Does it require a M. fervidus promoter sequence? Is transcription starting (and stopping) at specific locations? Can conditions be found where transcription is absolutely dependent on HMf?

d) Sequencing and comparison of M. fervidus genes. Sequencing the genes cloned from M. fervidus which encode hydrogenase(s) and rRNAs will be completed. The primary sequences obtained will be compared to functionally homologous sequences from mesophilic and moderately thermophilic methanogens. The potential for increased thermostability via increased secondary structure in 16SrRNA and 23SrRNAs will be sought. The cloned M. fervidus polyferredoxin-encoding gene (mvhB) will be subcloned into an expression system to facilitate high levels of synthesis in E. coli. Once expressed in E. coli it will be possible to purify and begin analyzing the structure-function relationships of this very unusual protein.

4) The following publications cite ONR support

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Training

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Joseph A. Krzycki: ONR Senior Research Associate: (7/88-9/88).

Kathleen M. Sandman: Postdoctoral Fellow (O.S.U. Fellowship): 9/88-present.

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Christopher J. Timan: ONR supported technician: 4/88-present.

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