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Title: Structure and Regulation of Methanogen Genes

Objective: Analysis of the products of the mcr operon in Methanococcus
vannielii.

i) Characterization of the transcript. Gene specific sense and antisense hybridization probes have been generated *in vitro* by transcription, using T7 RNA polymerase, of several regions of the *mcrBDCGA* operon subcloned into the T7 transcription vectors pT7-1 and pT7-2. Using these transcripts as probes we have demonstrated, by Northern blotting, that the *mcr* operon in *M. vannielii* is transcribed into a single, 5.1kb transcript that is not processed into discrete, smaller mRNAs. Transcription of the *mcr* operon has been shown to occur at all growth phases but to be maximal in cells in the late exponential phase of growth in batch cultures. It has been assumed that the *mcr* operon must be highly expressed but no comparative data were available to evaluate this assumption. We therefore constructed probes to identify and quantitate the mRNAs transcribed from the *M. vannielii* *secY*, *argG* and *mvaL10* genes. These probes revealed that the *mcr* operon is highly transcribed, at approximately the same level as the *mvaL10* gene which encodes the ribosomal protein L10 and at much higher levels than the *secY* gene (encodes a protein secretion function) and the *argG* gene (encodes argininosuccinate synthetase) (Table 1). The half-lives of these four mRNAs varied from 6.5 min. to 57 min. in *M. vannielii* cells growing exponentially at 37°C with a generation time of ~5h. Although there are approximately twice as many *mvaL10* transcripts per cell as transcripts of the *mcr* operon, the *mcr* transcript is almost three times the length of the *mvaL10* transcript. From these experiments we conclude that the *mcr* operon is highly expressed *in vivo*, generating a single-sized transcript with a half-life of ~15 min. at 37°. Our previous studies (Brown and Reeve, 1985, J. Bacteriol. 162:909) had shown that the average half-life for the total population of mRNAs in *M. vannielii* was 12

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min. We have now clearly demonstrated however that individual mRNAs have half-lives that differ substantially from this average value.

Table 1: Quantity and stability of mRNAs in cells of *M. vannielii* exponentially growing at 37°

Transcript	Half life (mins.)	Full-length transcripts per cell
<i>mcrBDCGA</i>	14.8	~150
<i>mvaL10</i>	30.0	~240
<i>secY</i>	6.5	~1
<i>argG</i>	57.0	~10

Having determined the levels of these transcripts in exponentially growing cells, we have begun to determine the effects of procedures which inhibit growth on transcription of these genes. Table 2 summarizes the results so far obtained.

Table 2: The effect of inhibiting growth on gene specific transcription.

Mechanism of growth inhibition	Metabolic function inhibited	Transcription response		
		<i>mcrBDCGA</i>	<i>mvaL10</i>	<i>secY</i>
Hydrogen removal	Substrate starvation	↓	↓	N.D.*
Addition of BES	Inhibition of methanogenesis	↓	↓	N.D.
Addition of puromycin	Inhibition of translation	↓↔↑	↑	↑
Addition of pseudomonic acid	Inhibition of translation	↓	↑	N.D.
Addition of virginiamycin	Inhibition of translation	↓	↓	N.D.
Addition of levulinic acid	Inhibition of Factor F420 synthesis.	↓↔↑	↓↔↑	N.D.
Addition of monensin	Inhibition of ATP synthesis	↓	↓	N.D.

*Not yet determined; ↓ decreased, ↑ increased, ↓↔↑ no change; growth inhibition was monitored by OD₆₀₀ measurement and conditions chosen which inhibited growth without causing cell lysis.

Directly inhibiting methanogenesis by hydrogen starvation, addition of bromoethane sulfonate (BES) or levulinic acid resulted in decreased *mcr*

Objective: Isolate and characterize the polyferredoxin and investigate its expression and function in *Methanobacterium thermoautotrophicum*.

i) Subcloning and expression of the *mvhB* gene in *E. coli* and purification of the polyferredoxin. The polyferredoxin encoding *mvhB* gene has been subcloned and expressed in *E. coli*, fused to the *lacZ* gene, resulting in a *gplacZ-mvhB* fusion protein. Antibodies raised against this protein have been used to identify and purify the polyferredoxin (*gpmvhB*) from growing cells of *M. thermoautotrophicum* strain ΔH . These experiments are described in detail in the attached preprint [Steigerwald, V.J., Pihl, T.D. and Reeve, J.N. 1991. PNAS, Submitted (1/92) for Publication]. The results obtained demonstrate that *gpmvhB* is synthesized only in growing cells, is located predominantly in the cytoplasm and contains a large number of Fe and S atoms. Antibodies raised against the *gplacZ-mvhB* did not inhibit metronidazole reduction (a generic assay for ferredoxin function) in crude extracts of *M. thermoautotrophicum*.

ii) Regulation of expression of the *mvh* operon. The goal of this project is to identify, quantitate and analyze the regulation of synthesis and decay of the transcript of the *mvhDGAB* operon which encodes the MV-hydrogenase and the polyferredoxin in *M. thermoautotrophicum*. Our inability to obtain sufficiently rapid lysis of *M. thermoautotrophicum* cells to allow intact mRNAs to be isolated has been a problem. All physical methods so far investigated (sonication, French pressure-cell, grinding) have proven to be inadequate. We have now isolated a cell-wall hydrolyzing enzyme from the related methanogen *Methanobacterium wolfei* which should digest the pseudomurein component of the cell wall of *M. thermoautotrophicum* to produce osmotically sensitive protoplasts. Lysis of these protoplasts should then be readily obtained by detergent addition and should generate experimentally useful mRNA preparations.

Objective: Development of a transformation system for *M. thermoautotrophicum*.

i) Evaluation of the *pac* gene. It has been demonstrated that methanogens are sensitive to puromycin and introduction of the *pac* gene from *Streptomyces alboniger* into *Methanococcus voltae* confers puromycin resistance on this

transcription. There does not therefore appear to be a compensating stimulation of expression of the *mcr* operon *in vivo* to counteract inhibitor-caused reduction in methanogenesis. A most surprising result from these inhibitor studies was that addition of puromycin or pseudomonic acid, both of which inhibit translation, resulted in a ~3-fold increase in the *mvaL10* transcript. Inhibition of protein synthesis must therefore either stimulate transcription of this ribosomal protein gene or prevent the decay of its mRNA *in vivo*. Puromycin addition also increased the level of the *secY* mRNA. Pulse-chase experiments are in progress to differentiate between the two alternative explanations for increased mRNA levels.

ii) Characterization of the *mcr* polypeptides. Rabbit antibodies have been raised against the purified methyl reductase holoenzyme (MR; containing the products of the *mcrB*, *mcrG* and *mcrA* genes, i.e. *gpmcrB*, *gpmcrG*, *gpmcrA*), against the isolated *gpmcrB* and against *gpmcrD* purified from an *E. coli* strain over-expressing the subcloned *mcrD* gene. Using these antibodies and purified gene products, in ELISAs and in Western blots, we have determined that there are ~30 molecules of MR holoenzyme per molecule of *gpmcrD* in exponentially growing cells of *M. vannielii*. Cell fractionations and EM immunogold localizations have revealed that both MR and *gpmcrD* are located predominantly in the cell cytoplasm and that they sediment as a complex, with a molecular mass of ~300kDa, when centrifuged through sucrose gradients. Adding the anti-*gpmcrD* antibodies to methanogenic reaction mixtures decreased the rate of methanogenesis *in vitro* by ~30% regardless of whether the CH₄ was being generated from CO₂ or from CH₃-S-CoM. Mixing *gpmcrD* with the anti-*gpmcrD* antiserum removed its inhibitory effect, presumably by titrating out the effective antibodies, however adding *gpmcrD* to the inhibited reaction mixtures did not reactivate methanogenesis. It appears, therefore, that although *gpmcrD* is not a subunit of MR *per se* it must play a role in methanogenesis in crude extracts *in vitro*. This conclusion is currently being further investigated.

methanogen. The *pac* gene, which encodes puromycin acetyl transferase, integrates into the methanococcal genome using homologous flanking sequences (Gernhardt, et. al., 1990. Mol. Gen. Genet. 221:273). For our purposes we need the *pac* gene to function on a plasmid and at ~65° in *M. thermoautotrophicum*. We have therefore constructed several recombinant plasmids containing the *pac* gene, the pME2001 replicon and replicons which function in *E. coli* and in *Bacillus stearothermophilus*. These confer puromycin resistance on *E. coli* but not, so far, on the thermophilic *B. stearothermophilus*, presumably because the half-life of puromycin acetyl transferase at 56° is only 8 min. We have attempted to obtain more thermostable variants by selecting for growth of *B. stearothermophilus* cells containing the plasmids with the *pac* gene on plates, at 56°, containing puromycin but spontaneously puromycin-resistant strains have yet to be recovered.

ii) Construction of pseudomonic acid resistance conferring vectors. Pseudomonic acid inhibits isoleucyl-tRNA synthetase and mutants of *M. thermoautotrophicum* strain Marburg, resistant to pseudomonic acid, have been isolated. The gene (*ileS*) encoding the isoleucyl tRNA synthetase from one of these mutants has been cloned and sequenced and the pseudomonic acid resistance shown to result from a single base change in this gene (Jenal et al., 1991. J. Biol. Chem. 266:10570). We have constructed a variety of recombinant plasmids which carry this mutated *ileS* gene, the plasmid pME2001 replicon and the promoter from the highly expressed *mvh* operon. Transformation of *M. thermoautotrophicum* using electroporation protocols, and these plasmids as the donor DNA will be investigated.

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