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W. B. Whitman: Progress Report
Grant No. DE-FG05-94ER20158
Biochemistry and Genetics of Autotrophy in Methanococcus

During the first two years of this project, one paper was published describing the transformation of *Methanococcus maripaludis*: Tumbula, D.L., Makula, R.A., and Whitman, W.B. 1994. Transformation of *Methanococcus maripaludis* and identification of a *Pst*I-like restriction system. FEMS Microbiol. Lett. 121: 309-314. Three papers were presented at the National Meeting of the American Society for Microbiology: Yang, Y.L., and Whitman, W.B. 1994. Purification and characterization of pyruvate oxidoreductase from *Methanococcus maripaludis*. Abst. Ann. Meet. Am. Soc. Microbiol. 94: 287; Tumbula, D.T., and Whitman, W.B. 1995. Plasmid vector developments in *Methanococcus maripaludis*. Abst. Ann. Meet. Am. Soc. Microbiol. 95: 320; and Tumbula, D.L., and Whitman W.B. 1996. A replicating shuttle vector for *Methanococcus maripaludis*. Abst. Ann. Meet. Am. Soc. Microbiol. 96: 306. Reprints and copies of the abstracts are enclosed. Three manuscripts are also in preparation: Yang, Y.L., and Whitman, W.B. Purification and characterization of the anabolic pyruvate oxidoreductase from *Methanococcus maripaludis*; Yang, Y.L., Tumbula, D.L., Gluska, J.N., Teng, Q., and Whitman, W.B. In vivo isotopic exchange reactions and the role of pyruvate oxidoreductase in *Methanococcus maripaludis*; and Tumbula, D.L., and W.B. Whitman. Characterization of the methanococcal plasmid pURB500 and development of a shuttle vector. Progress in some of these areas of research are described in more detail below.

Development of a shuttle vector. In the last two years, the most exciting results have come from the work on the genetics of methanococci. First, we demonstrated that the cryptic plasmid from *Methanococcus maripaludis* C5, pURB500, could be transformed into *Methanococcus maripaludis* JJ. Strain JJ is the type strain of *M. maripaludis* and has only about 65 % DNA:DNA hybridization to strain C5 (Keswani et al., 1996). Because of the low relatedness of these strains, it was not obvious that pURB500 could be transferred between them. This goal was achieved by first transforming strain C5 with a series of suicide plasmids containing the *pac* cassette, which possessed the selectable puromycin resistance marker (Gernhardt et al., 1990), and different cloned fragments of pURB500. From the puromycin-resistant transformants, a plasmid was isolated that transformed strain JJ. However, when this plasmid was electroporated into *E. coli*, only rearrangement products were obtained that contained small portions of the original pURB500. These plasmids no longer transformed *Methanococcus*. While these experiments did not yield a shuttle vector, they demonstrated that pURB500 could replicate in strain JJ.

Based upon these results, pMEB.2, which contains the *pac* cassette in a pUC-based *E. coli* plasmid, was cloned into a partial EcoRI digest of the methanococcal plasmid pURB500. After transformation of *M. maripaludis* JJ, a hybrid plasmid was isolated which contained the pMEB.2 insertion at one of the four possible *Eco*RI sites (Figure 1). This plasmid, pDLT44, was electroporated into *E. coli* by selecting for the ampicillin resistance marker on the pUC-component. When prepared from *E. coli*, pDLT44 could be transformed back into *M. maripaludis* at a high frequency (Table 1). Thus, it has the features necessary for a shuttle vector between the methanococci and *E. coli*.

Subsequent examination of pTLB44 revealed that it was rapidly lost from *E. coli* in the absence of the ampicillin selection. Variants of the plasmid that contained only portions of the original pURB500 were more stable in *E. coli* but were unable to transform *Methanococcus*. These results indicate that pTLB44 contains one or more regions that are toxic to *E. coli* but are necessary for replication in *Methanococcus* and explain the initial difficulties in obtaining the shuttle vector.

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Table 1. PEG transformation of *M. maripaludis* and *E. coli* by the shuttle vector pDLT44. pDLT44 transformed *M. maripaludis* JJ and *E. coli* at high frequencies but not other species of mesophilic methanococci. However, a positive control (pMip1) was available only for *M. voltae*, so the failure of the other species to transform could be due to technical problems.

host transformed	plasmid[a]	transformants/ug	transformants/CFU
E. coli XL1-Blue MRF[b]	pDLT44	4.0×10^{8}	nd[c]
M. maripaludis JJ	pDLT44	4.6×10^{7}[d]	4.7×10^{-4}
M. voltae PS	pDLT44	<89	$<9.4 \times 10^{-8}$
M. voltae PS	pMip1	94	8.8×10^{-4}
M. vannielii SB	pDLT44	<89	$<1.1 \times 10^{-8}$
M. aeolicus PL-15/H	pDLT44	<89	$<1.7 \times 10^{-4}$

[a]Plasmid DNA was purified from *E. coli* except for the pDLT44 used to transform *E. coli*, which was purified from *M. maripaludis* JJ.
[b]d(*mcrA*)183, d(*mcrCB-hsdSMR-mrr*)173, *endA1*, *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, *lac*, [F' *proAB*, *lacI*^{q}*ZdM15*, Tn*10* (Tet^{r})].
[c]Not determined.
[d]Average of two experiments.

The plasmid pURB500 has also been sequenced in its entirety. The 8285 bp plasmid contains two regions of complex secondary structure at positions 4000-4400 and 5700-6000. In addition, four open reading frames (ORF) greater than 180 amino acids and upto 14 smaller ORFs are present (Figure 1). Because of their location and the direction of translation, some of these ORFs could compose an operon. However, there is no direct evidence concerning the expression of the ORFs and the function of the potential secondary structures.

Isolation of acetate auxotrophs. In addition, the acetyl-CoA decarbonylase/synthase (ACDS, formerly carbon monoxide dehydrogenase) of *M. maripaludis* was highly purified on the basis of the dye reduction assay. Isotope exchange activity was rapidly lost during the purification, and the purification was hindered by the low abundance of the enzyme in whole cells. Although the yields of active enzyme were low, sufficient material was obtained for N-terminal sequence analysis. From this sequence data and comparison of the sequences of the homologous *Methanosarcina*, *Methanosaeta*, *Clostridium*, and *Rhodospirillum* enzymes, degenerate primers were designed for PCR amplification of the *M. maripaludis* genes. The PCR products were then used to screen a cosmid library, and three clones, labeled 712, 150, and 222, were identified that hybridized to two or more of the PCR products.

To examine the biological affect of 712, it was subcloned into pMEB.2 to form p712, *M. maripaludis* was transformed with p712, and puromycin resistant colonies were selected. The rationale for this experiment was that insertion of p712 into the genome by homologous recombination should disrupt expression of the ACDS gene(s) and produce an acetate auxotrophy. Preliminary experiments suggested that the transformants did indeed grow poorly in the absence of acetate. However, sequencing of 712 did not reveal ORFs homologous to the ACDS genes.

More thorough characterization of the growth properties of the transformant suggested that the acetate stimulation was probably due to the requirement of the resistance marker, puromycin

transacetylase from *Streptomyces*, for high levels of acetyl-CoA. Thus, it might be possible for any mutation that disrupts cellular function so that the levels of intracellular acetyl-CoA are lowered to have a large affect on growth in the presence of puromycin. In support of this hypothesis, the growth of a transformant containing pKAS102 (Sanbeck and Leigh, 1991), which is not believed to have a direct affect on acetate biosynthesis, is also stimulated by acetate (Figure 2A). Moreover, 712 was also transformed into *M. maripaludis* in the phagemid vector pBK-CMV. In this vector, a neomycin resistance gene is under control of the SV40 early promoter, which should be active in methanococci. Neomycin-resistant transformants grew poorly in both the presence and absence of acetate, and the acetate-specific effect was reduced (Figure 2B). These results suggest that p712 insertion may cause a pleiotrophic mutation that is not directly related to acetate biosynthesis.

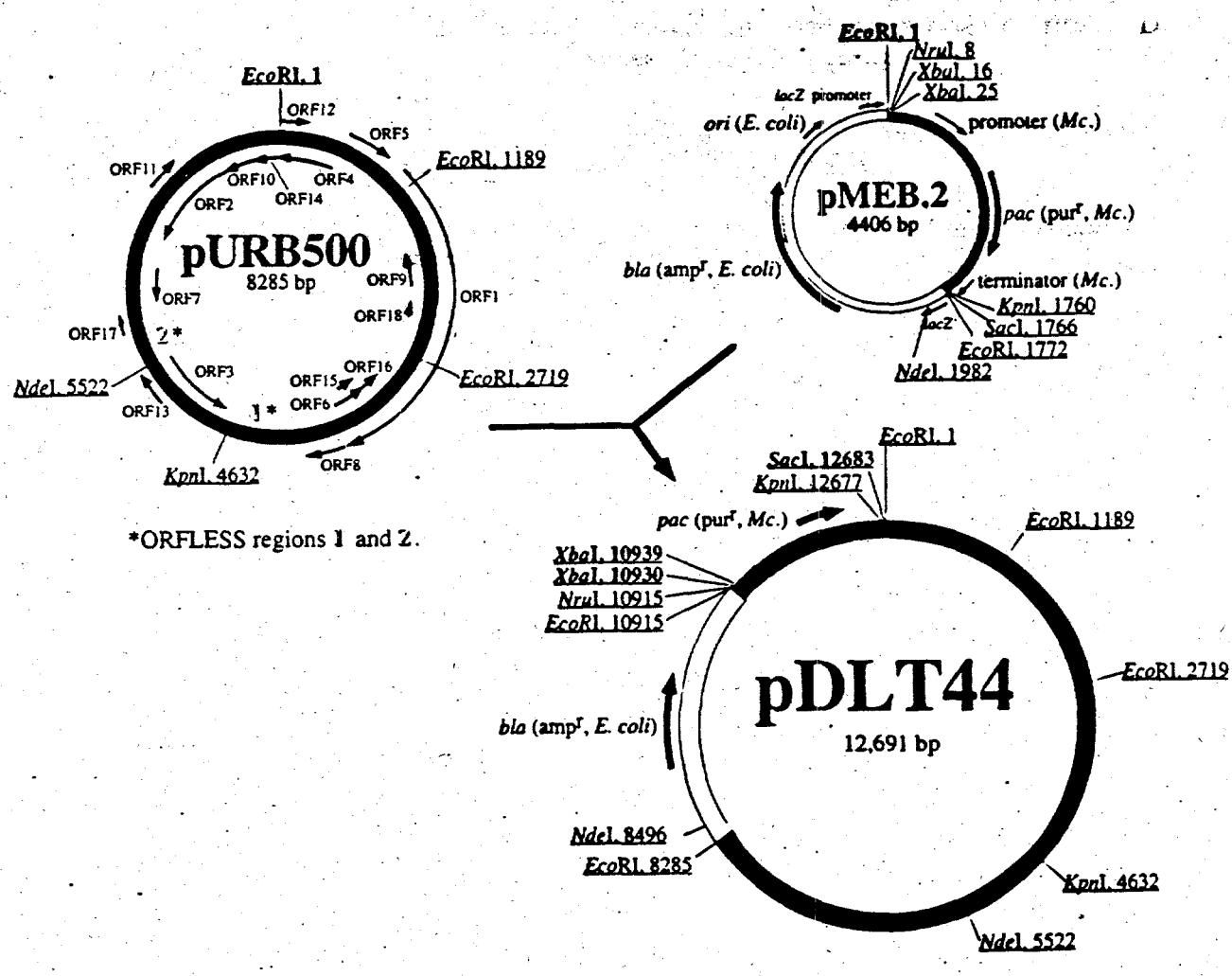

Figure 1. Construction of the shuttle vector pDLT44 from the methanococcal plasmid pURB500 and pMEB.2, which carries the puromycin resistance cassette *pac*.

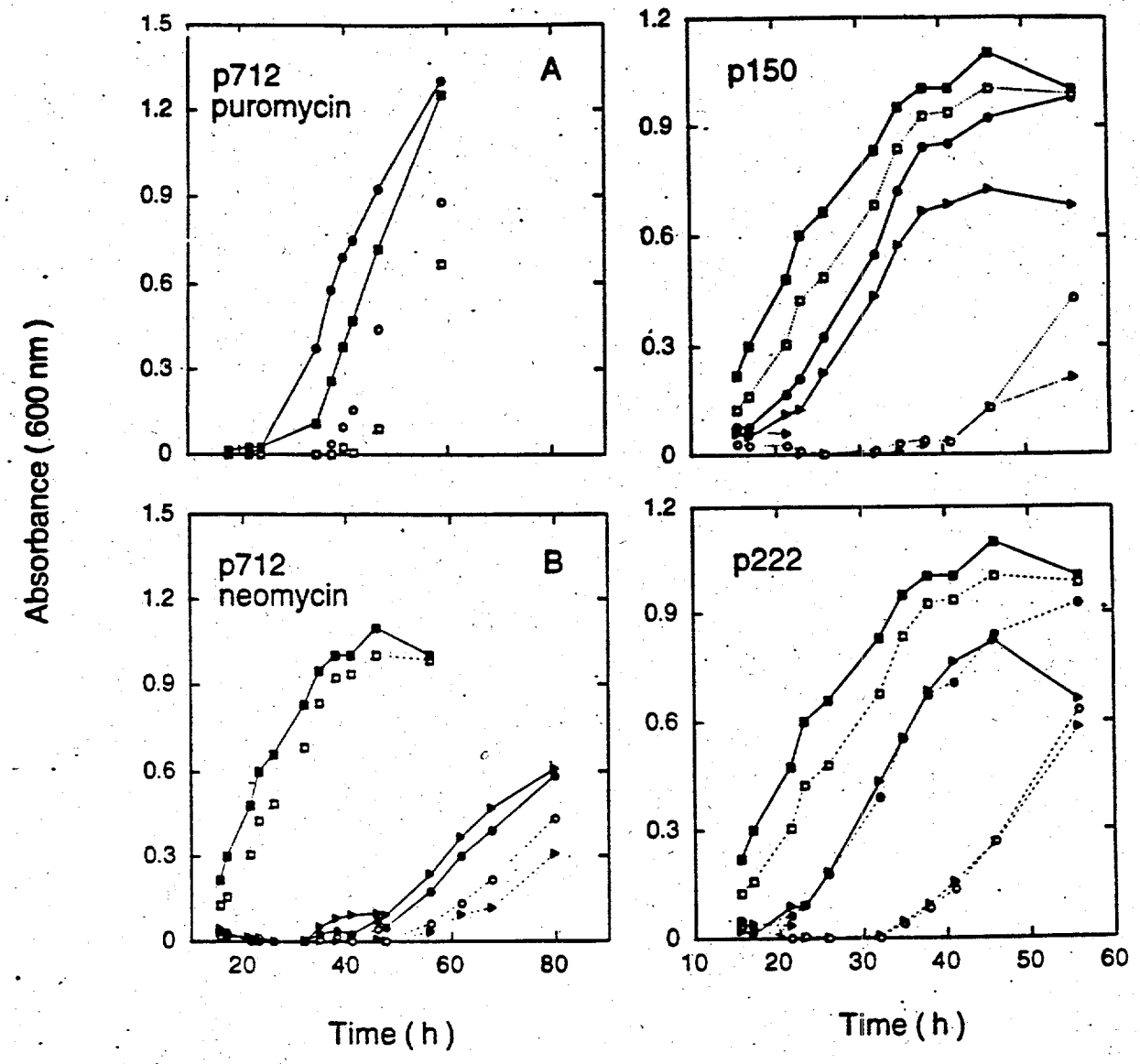

Figure 2. Acetate stimulation of growth of transformants containing p712. Open symbols: growth in mineral (McN) medium. Closed symbols: growth in McN+10 mM sodium acetate. A. Growth of transformants containing the puromycin resistance marker in medium with 2.5 µg/ml puromycin. (●,○) transformants of p712; (■,□) transformants of pKAS102. B. Growth of transformants of p712 containing the neomycin marker. (■,□) Growth of the wild-type strain JJ1 without neomycin; (●,○) growth of the transformant without neomycin; (▲,△) growth of the transformant with 1 mg/ml neomycin.

Figure 3. Acetate stimulation of growth of neomycin-resistant transformants of p150 and p222. Open symbols: growth in McN. Closed symbols: McN+acetate. (■,□) Growth of wild-type strain JJ1 without neomycin; (●,○) growth of transformant without neomycin; (▲,△) growth of transformant with 1 mg/ml neomycin.

Similar experiments were performed with the other two clones, 150 and 222. Using the phagemid vector and the neomycin resistance marker, transformants grew poorly in the absence of acetate (Figure 3). The presence of neomycin had no affect on this growth requirement. The major affect seemed to on the culture lag and not the exponential growth rate (Table 2). Moreover, the lag was reproducible even after serial transfers in medium without acetate. Thus, these transformants appear to be leaky acetate auxotrophs, presumably formed by insertional inactivation of some chromosomal genes. Further characterization of these auxotrophs are in progress to determine the actual modification. Additional growth experiments will be performed to delineate the nutritional basis for the poor growth. Using Southern hybridizations, an insertional mutation will be demonstrated. Sequencing of the inserts will be completed to determine the nature of the target gene.

Table 2. Growth properties of neomycin-resistant transformants of p150 and p222. Culture media did not contain neomycin, but results with 1 mg/ml neomycin were essentially the same[a]

strain	+/- acetate	doubling time (h)	lag time (h)
wild-type	-	2.8	12
	+	3.3	9
p150	-	5.5	41
	+	4.3	17
p222	-	5.0	38
	+	3.5	21
p712	-	8.8	56
	+	7.3	43

[a]All results are the averages of duplicate experiments.

Isotope labeling studies. Two studies were completed concerning pyruvate metabolism in *Methanococcus maripaludis*. In the first study, the intracellular pyruvate pool of growing cells was successfully labeled with 18% [U-^{13}C]alanine. By examining the labeling pattern of leucine and valine, this experiment allowed the measurement of the in vivo conversion of pyruvate to acetyl-CoA, the pyruvate:CO_2 exchange reaction, and the acetyl-CoA:CO_2 exchange reaction (Figure 4 and data not shown). The C3, C4, C5, and C6 of leucine were derived from two molecules of pyruvate, and the enrichment at each position was about 16 %, which was close to the medium alanine enrichment. Thus, the intracellular pyruvate pools were labeled quantitatively. The spectra of C4 and C5 contained large doublets because each resonance is split by a neighboring ^{13}C atom. This pattern was consistent with both positions being derived from the same molecule of ^{13}C-alanine. Likewise, a large singlet dominated the spectra of C3 and C6. This pattern was expected due to migration of a methyl group during biosynthesis. After incorporation into the intermediate acetolactate, the carbon atoms from one of the molecules of pyruvate are separated. Because of the low enrichment of alanine in the medium, most ^{13}C atoms are placed next to a ^{12}C atom, and their resonances would not be split. The small doublets at C3 and C6 then represent splitting due to the enrichment of the second molecule of pyruvate. In contrast,

the enrichment of the C1 and C2 of leucine, which were derived from acetyl-CoA, was much lower (Figure 4). Thus, pyruvate was not a major source of acetyl-CoA under these growth conditions. The doublets for C1 and C2 were due to incorporation from a single molecule. However, the central singlet for C2 was enriched relative to the singlet of C1. This result suggests that label was selectively removed from the C1 position, presumably by the acetyl-CoA:CO_2 exchange reaction catalyzed by ACDS. Thus, even under conditions where most of the cellular carbon is obtained from alanine via pyruvate, autotrophic acetyl-CoA was still the favored route of acetyl-CoA biosynthesis. To determine the fate of carbon derived from the C1 of alanine, valine was also examined (data not shown). In this experiment, no enrichment of the C1 of valine was observed even though the enrichment of other carbons was comparable to leucine. This result suggested that label in the C1 of pyruvate was quantitatively removed by the pyruvate:CO_2 exchange reaction catalyzed by POR. In conclusion, only a small portion of the intracellular pyruvate was metabolized to acetyl-CoA, and the C1 of pyruvate was quantitatively exchanged with CO_2. These results also appear to preclude a major role for pyruvate as a C-1 donor in autotrophic acetyl-CoA biosynthesis in vivo because, in this case, the enrichment of acetyl-CoA would be one-half of that of pyruvate.

Moreover, the pyruvate:CO_2 exchange reaction may have important consequences for the interpretation of isotope tracer studies in methanococci. For instance, Choquet et al. (1994) proposed a novel pathway of erythrose-4-phosphate (E4P) biosynthesis based in part on the incorporation of [1-^{13}C]pyruvate. E4P is an intermediate in pentose and aromatic amino acid biosynthesis. However, careful examination of their methods suggested that the level of incorporation was very close to the levels expected if [1-^{13}C]pyruvate was exchanged with the carbonate in the medium and $^{13}CO_2$ was the actual species incorporated. Therefore, we performed a second study to examine the distribution of label from highly enriched [2-^{13}C]acetate. In this study, an acetate auxotroph was utilized, and the incorporation into cytidine was measured (Figure 5). In this experiment, the enrichment was determined by examining the splitting of the proton resonances linked to the ^{13}C atoms. The incorporation at position 5, which was derived entirely from the C2 of acetate, was compared to that at position 2', which represented one of the carbons of ribose. Choquet's pathway predicted that 50% of the label at position 2' would be derived from acetate. A pathway using established enzymes (Yu et al., 1994) predicted that 66%-85% of the label would be derived from acetate, where the higher value represented the case were about 1/3 of the E4P was utilized for aromatic amino acid biosynthesis and 2/3 for pentose biosynthesis and the lower value represented the case were E4P was only utilized for pentose biosynthesis. For the H-5, the enrichment observed was 95%, calculated by comparing the intensity of the central doublet, which represents a H atom bonded to ^{12}C atoms, to the two flanking doublets, which represented H atoms bonded to ^{13}C atoms (Figure 5). For the H-2', the measured enrichment was 64%. After correcting for the low enrichment at position 5, this value became 67%. Two explanations seem possible. One, both pathways are utilized, and the labeling pattern is an average. Two, E4P is not utilized for aromatic amino acid biosynthesis in methanococci. Although there is little precedent for alternative pathways of aromatic amino acid biosynthesis in microorganisms, it is curious that DAHP synthase, the first enzyme in the pathway, was not detected in *Methanohalophilus mahii* (Fischer et al., 1993). We hope that the genome sequence of *Methanococcus jannaschii* will provide additional insight into this question. The genes for a large number of DAHP synthases have been sequenced. If this enzyme is present in methanococci, its gene should be readily detected.

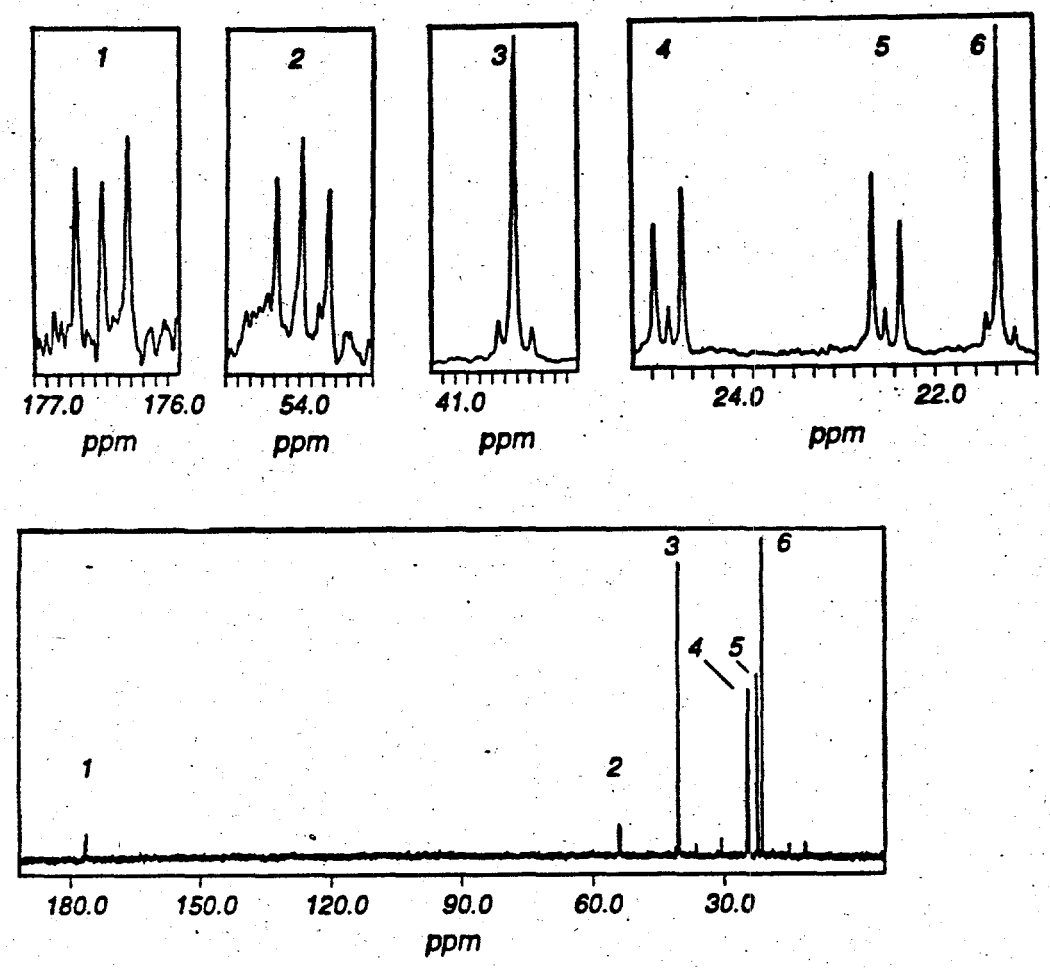

Figure 4. ^{13}C-NMR of leucine derived from *M. maripaludis* following growth with 18% [U-^{13}C]alanine as the sole nitrogen source. In the upper panels, the resonances are expanded to show the satellite peaks and the relative intensities at positions 1 and 2 are increased 6.5-fold.

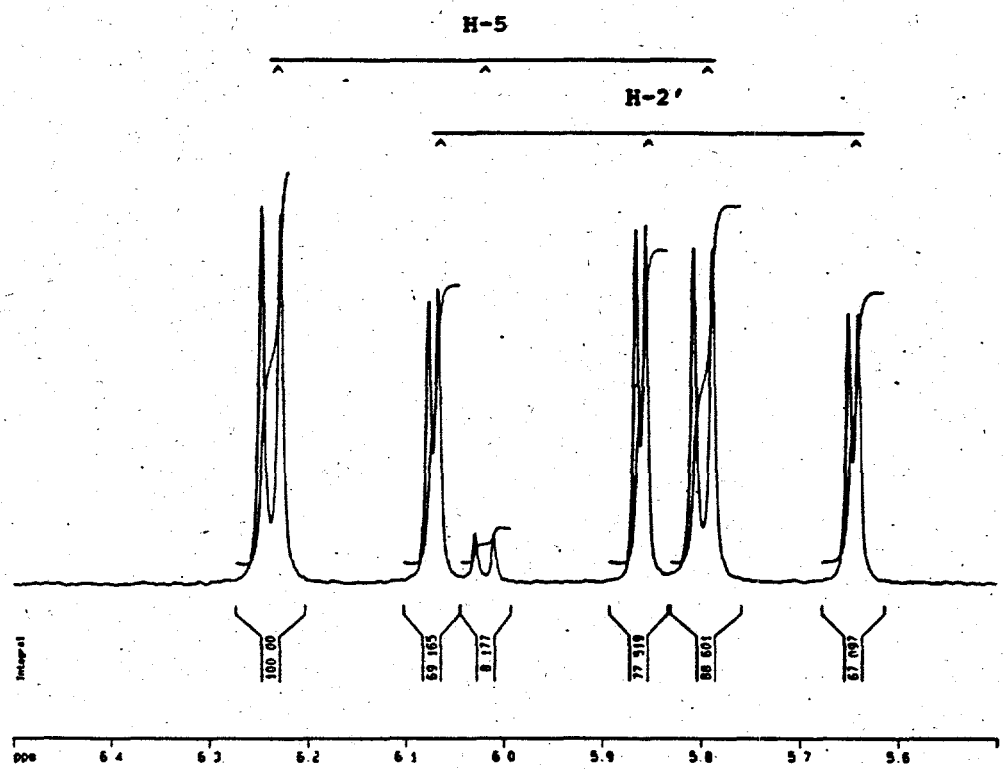

Figure 5. Proton NMR of the H-5 and H-2' of cytidine from *M. maripaludis* JJ6 following growth with [2-^{13}C]acetate. The carbon at position 5 of the pyrimidine should be derived entirely from the C2 of acetate. The carbon at position 2' of the ribose should be derived only in part from the C2 of acetate.

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2. Selkov, E., N. Maltsev, G.J. Olsen, R. Overbeek, C.R. Woese, and W.B. Whitman (1997). A reconstruction of the metabolism of *Methanococcus jannaschii* from sequence data. GENE COMBIO., 197:GC10-25.
3. Tumbula, D.L., T.L. Bowen, and W.B. Whitman (1997). Characterization of pURB500 from the archaeon *Methanococcus maripaludis* and construction of a shuttle vector. J. Bacteriol., 179:2976-2986.
4. Whitman, W.B., D.L. Tumbula, J.P. Yu, and W. Kim (1997) Development of genetic approaches for the methane-producing archaebacterium *Methanococcus maripaludis.* BioFactors 6:37-46.
5. Tumbula, D.L., Q. Teng, M.B. Bartlett, and W.B. Whitman (1997) Ribose biosynthesis and evidence for an alternative first step in the common aromatic amino acid pathway in *Methanococcus maripaludis.* J. Bacteriol. 179:6010-6013.

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Isotope labeling studies. Two studies were completed concerning pyruvate metabolism in *Methanococcus maripaludis*. In the first study, the intracellular pyruvate pool of growing cells was successfully labeled with 18% [U-^{13}C]alanine. By examining the labeling pattern of leucine and valine, this experiment allowed the measurement of the in vivo conversion of pyruvate to acetyl-CoA, the pyruvate:CO_2 exchange reaction, and the acetyl-CoA:CO_2 exchange reaction. The C3, C4, C5, and C6 of leucine were derived from two molecules of pyruvate, and the enrichment at each position was about 16 %, which was close to the medium alanine enrichment. Thus, the intracellular pyruvate pools were labeled quantitatively. The spectra of C4 and C5 contained large doublets because each resonance is split by a neighboring ^{13}C atom. This pattern was consistent with both positions being derived from the same molecule of ^{13}C-alanine. Likewise, a large singlet predominated the spectra of C3 and C6. This pattern was expected due to migration of a methyl group during biosynthesis. After incorporation in the intermediate acetolactate, the carbon atoms of one of the molecules of pyruvate are separated. Because of the low enrichment of alanine in the medium, most ^{13}C atoms would be placed next to a ^{12}C atom, and their resonances would not be split. The small doublets at C3 and C6 then represent splitting due to the enrichment of the second molecule of pyruvate. In contrast, the enrichment of the C1 and C2 of leucine, which were derived from acetyl-CoA, was much lower. Thus, pyruvate was not a major source of acetyl-CoA under these growth conditions. The doublets for C1 and C2 were due to incorporation from a single molecule. However, the central singlet for C2 was enriched relative to the singlet of C1. This result suggests that label was selectively removed from the C1 position, presumably by the acetyl-CoA:CO_2 exchange reaction catalyzed by ACDS. Thus, even under conditions where most of the cellular carbon is obtained from alanine via pyruvate, autotrophic acetyl-CoA was still the favored route of acetyl-CoA biosynthesis. To determine the fate of carbon derived from the C1 of alanine, valine was also examined (data not shown). In this experiment, no enrichment of the C1 of valine was observed even though the enrichment of other carbons was comparable to leucine. This result suggested that label in the C1 of pyruvate was quantitatively removed by the pyruvate:CO_2 exchange reaction

catalyzed by POR. In conclusion, only a small portion of the intracellular pyruvate was metabolized to acetyl-CoA, and the C1 of pyruvate was quantitatively exchanged with CO_2. These results also appear to preclude a major role for pyruvate as a C-1 donor in autotrophic acetyl-CoA biosynthesis in vivo because, in this case, the enrichment of acetyl-CoA would be one-half of that of pyruvate.

Moreover, the pyruvate:CO_2 exchange reaction may have important consequences for the interpretation of isotope tracer studies in methanococci. For instance, Choquet et al. (1994) proposed a novel pathway of erythrose-4-phosphate (E4P) biosynthesis based in part on the incorporation of [1-^{13}C]pyruvate. E4P is an intermediate in pentose and aromatic amino acid biosynthesis. However, careful examination of their methods suggested that the level of incorporation was very close to the levels expected if [1-^{13}C]pyruvate was exchanged with the carbonate in the medium and $^{13}CO_2$ was the actual species incorporated. Therefore, we performed a second study to examine the distribution of label from highly enriched [2-^{13}C]acetate. In this study, an acetate auxotroph was utilized, and the incorporation into cytidine was measured. In this experiment, the enrichment was determined by examining the splitting of the proton resonances linked to the ^{13}C atoms. The incorporation at position 5, which was derived entirely from the C2 of acetate, was compared to that at position 2′, which represented one of the carbons of ribose. Choquet's pathway predicted that 50% of the label at position 2′ would be derived from acetate. A pathway using established enzymes (Yu et al., 1994) predicted that 66%-85% of the label would be derived from acetate, where the higher value represented the case were about 1/3 of the E4P was utilized for aromatic amino acid biosynthesis and 2/3 for pentose biosynthesis and the lower value represented the case were E4P was only utilized for pentose biosynthesis. For the H-5, the enrichment observed was 95%, calculated by comparing the intensity of the central doublet, which represents a H atom bonded to ^{12}C atoms, to the two flanking doublets, which represented H atoms bonded to ^{13}C atoms. For the H-2′, the measured enrichment was 64%. After correcting for the low enrichment at position 5, this value became 67%. Two explanations seem possible. One, both pathways are utilized, and the labeling pattern is an average. Two, E4P is not utilized for aromatic amino acid biosynthesis in methanococci. Although there is little precedent for alternative pathways of aromatic amino acid biosynthesis in microorganisms, it is curious that DAHP synthase, the first enzyme in the pathway, was not detected in *Methanohalophilus mahii* (Fischer et al., 1993). We hope that the genome sequence of *Methanococcus jannaschii* will provide additional insight into this question. The genes for a large number of DAHP synthases have been sequenced. If this enzyme is present in methanococci, its gene should be readily detected.

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