Isolation of Acetate Auxotrophs of the Methane-Producing Archaeon *Methanococcus maripaludis* by Random Insertional Mutagenesis

Wonduck Kim and William B. Whitman

Department of Microbiology, University of Georgia, Athens, Georgia 30602-2605

Manuscript received March 23, 1999
Accepted for publication May 5, 1999

ABSTRACT

To learn more about autotrophic growth of methanococci, we isolated nine conditional mutants of *Methanococcus maripaludis* after transformation of the wild type with a random library in pMEB.2, a suicide plasmid bearing the puromycin-resistance cassette *pac*. These mutants grew poorly in mineral medium and required acetate or complex organic supplements such as yeast extract for normal growth. One mutant, JJ104, was a leaky acetate auxotroph. A plasmid, pWDK104, was recovered from this mutant by electroporation of a plasmid preparation into *Escherichia coli*. Transformation of wild-type *M. maripaludis* with pWDK104 produced JJ104-1, a mutant with the same phenotype as JJ104, thus establishing that insertion of pWDK104 into the genome was responsible for the phenotype. pWDK104 contained portions of the methanococcal genes encoding an ABC transporter closely related to MJ1367-MJ1368 of *M. jannaschii*. Because high levels of molybdate, tungstate, and selenite restored growth to wild-type levels, this transporter may be specific for these oxyanions. A second acetate auxotroph, JJ117, had an absolute growth requirement for either acetate or cobalamin, and wild-type growth was observed only in the presence of both. Cobinamide, 5′,6′-dimethylbenzimidazole, and 2-aminopropanol did not replace cobalamin. This phenotype was correlated with tandem insertions in the genome but not single insertions and appeared to have resulted from an indirect effect on cobamide metabolism. Plasmids rescued from other mutants contained portions of ORFs denoted in *M. jannaschii* as endoglucanase (MJ0555), transketolase (MJ0681), thiamine biosynthetic protein *thiI* (MJ0931), and several hypothetical proteins (MJ1031, MJ0835, and MJ0835.1).

METHANOCOCCUS maripaludis is a marine, methanogenic archaeon that utilizes $H_2 + CO_2$ or formate as sole carbon and energy sources (Jones *et al.* 1983). During autotrophic growth, CO_2 is assimilated by a modified Ljungdahl-Wood pathway of acetyl-CoA biosynthesis (Whitman 1994). In this pathway, methyltetrahydromethanopterin produced during methanogenesis donates a methyl group to the corrinoid Fe/S protein of the acetyl-CoA decarbonylase/synthase complex (ACDS, also called CO dehydrogenase). This complex reduces CO_2 to the oxidation level of CO and combines it with HS-CoA and the methyl group to form acetyl-CoA. In *M. maripaludis*, the biosynthetic activity as well as a number of diagnostic partial activities of the ACDS complex have been detected in cell-free extracts (Shieh and Whitman 1988). In addition, acetate auxotrophs isolated following ethyl methanesulfonate mutagenesis had low levels of ACDS activity, and spontaneous revertants recovered wild-type levels of ACDS activity (Ladapo and Whitman 1990). While this evidence is indicative of a role for the ACDS complex, the complex has never been isolated from an autotrophic methanogen, and its role in autotrophy is largely inferred from the properties of the enzyme in the homoacetogenic clostridia and aceticlastic methanogens.

To identify additional factors important for autotrophy in *M. maripaludis*, random insertional mutagenesis was performed to isolate mutants unable to grow autotrophically. In this approach, cells were transformed with a genomic library in pMEB.2, a pUC derivative bearing the puromycin-resistance cassette *pac* (Gernhardt *et al.* 1990). This plasmid is unable to replicate in methanococci, and puromycin resistance can be acquired only by homologous recombination into the genome at the site of the cloned genomic DNA (Sandbeck and Leigh 1991). If the cloned fragment is internal to a gene or operon, integration will disrupt expression of the gene and/or downstream genes. While similar techniques have been utilized to inactivate specific genes in the methanococci (*e.g.*, Berghofer and Klein 1995; Jarrell *et al.* 1996), this is the first report of its application to random mutagenesis.

MATERIALS AND METHODS

Strains: The wild-type strain of *M. maripaludis*, strain JJ, was obtained from W. J. Jones (Jones *et al.* 1983). *Escherichia coli* SURE was obtained from Stratagene (La Jolla, CA).

Media and culture conditions: *M. maripaludis* was grown in mineral medium (McN) at 37° with $H_2 + CO_2$ as the carbon and energy source as described previously (Whitman *et al.* 1986). Unless specified otherwise, 5-ml cultures were grown

Corresponding author: William B. Whitman, Department of Microbiology, University of Georgia, Athens, GA 30602-2605.
E-mail: whitman@arches.uga.edu

in 28-ml stoppered tubes using strictly anaerobic procedures (Balch *et al.* 1979). The initial gas pressure was 240 kPa, and tubes were repressurized two or three times a day. McA was composed of McN plus 10 mm sodium acetate. McC was composed of McN plus 0.2% (w/v) yeast extract. McAC was composed of McA plus 0.2% yeast extract. McAC$^+$ was composed of McAC plus 0.2% (w/v) Casamino acids, 1% (v/v) vitamin solution (Whitman *et al.* 1986), 0.15% (w/v) each of valine, isoleucine, and leucine, and 0.01 mm pantoyllactone. Growth on agar plates was performed as described previously (Tumbula *et al.* 1995a). Solid medium had the same composition as broth except that the $NaHCO_3$ concentration was reduced to 2 g liter^{-1} and 1% (w/v) Noble agar (Difco, Detroit) was added. Replica plating was performed with sterile toothpicks that had been incubated in the anaerobic chamber for 1 day. Growth of 200-ml cultures was performed in 1-liter bottles and McAC medium as described previously (Balch *et al.* 1979).

For cleaning, culture tubes, stoppers, and flasks were autoclaved in 0.2 n NaOH for 20 min. After cooling, the glassware and stoppers were rinsed in tap water followed by deionized water.

Construction of library: A 200-ml culture of *M. maripaludis* was grown overnight and harvested by centrifugation for 15 min at 7300 × *g* at 4°. The cell pellet was washed in 30 ml of 0.4 m NaCl and resuspended in 3 ml of 0.15 m NaCl + 0.1 m sodium EDTA, pH 8.0. To lyse the cells, the cell suspension was placed in a −70° freezer. The suspension was then thawed in a water bath at room temperature, and 5 ml of phenol-chloroform-isoamyl alcohol extraction solution was added (Saito and Miura 1963). After gently mixing, the solution was centrifuged for 10 min at 8200 × *g* at 4°. The aqueous phase was then recentrifuged for 20 min at 8200 × *g* at 4° to remove precipitated sulfide and protein. Finally, the DNA was collected by ethanol precipitation and washed with 70% ethanol (Sambrook *et al.* 1989). After drying, the pellet was resuspended in a minimum volume of TE buffer (10 mm Tris chloride, pH 8.0, and 1 mm sodium EDTA, pH 8.0) at 4°.

To remove RNA, the DNA was treated with 1 μl of DNase-free RNase (Sambrook *et al.* 1989) for 1 day at 4°. To prepare DNA fragments, 10 μl of DNA (3 μg μl^{-1}) was diluted into 90 μl of 0.1× SSC, where SSC was 0.15 m NaCl and 15 mm sodium citrate, pH 7.0. The solution was then sonicated at 0° for 12 min in intervals of 0.5 min. For partial repair, 11 μl of the DNA solution was treated with 10 units of Klenow fragment (10 units μl^{-1}, Promega, Madison, WI) and 8 μl of 0.5 mm deoxynucleotide triphosphates. After 15 min at 30°, 3 μl of 0.5 m sodium EDTA (pH 8.0) was added to terminate the reaction. The vector pMEB.2 (Gernhardt *et al.* 1990) was digested with *Eco*RV and dephosphorylated with calf intestinal alkaline phosphatase (Sambrook *et al.* 1989). For ligation, 75 ng of the dephosphorylated vector and 23 ng of the partially repaired genomic DNA were combined with 200 units of T4 DNA ligase (Promega) and buffer in a total volume of 20 μl and incubated overnight at room temperature. After ligation, the DNA solution was diluted with four parts of water, and 1 μl was electroporated into *E. coli* SURE. The electroporation was repeated 10 times. The transformants were pooled, diluted to 50 ml in Luria-Bertani (LB) broth plus ampicillin (120 μg ml^{-1}), and incubated for 8 hr at 37°. After growth, the cell suspension was diluted with 50 ml of LB broth + 30% (v/v) glycerol, and 1-ml portions were stored at −70°. The total number of transformants obtained was 3×10^5, 75% of which contained cloned DNA.

To transform *M. maripaludis*, 0.1 ml of the frozen cell suspension was inoculated into 10 ml of LB broth + ampicillin (50 μg ml^{-1}). After growth overnight at 37°, the plasmids were purified from 1.5 ml of culture with Wizard Plus Miniprep systems (Promega). Purified plasmid, 3 μg in 40 μl of deionized water, was diluted with 60 μl of TE buffer in a microfuge tube. The plasmid preparation was then transferred to the anaerobic chamber and incubated for 1 day to allow O_2 to diffuse from the solution. Upon transformation of a 5-ml culture of *M. maripaludis*, the transformants were inoculated into 20 ml of McAC$^+$ containing 2.5 μg puromycin ml^{-1} in a 160-ml serum bottle and incubated for 2 days under $H_2 + CO_2$ at 37° to allow for growth. The culture was then dispensed into four sterile culture tubes and centrifuged at 2000 × *g* for 20 min at room temperature, and the cells were resuspended in a one-fifth volume of McC + 25% glycerol (Tumbula *et al.* 1995b). One-milliliter portions of the glycerol-containing cell suspensions were stored at −70°.

Transformation, plasmid purification, electrophoresis: *M. maripaludis* was transformed by the polyethylene glycol method of Tumbula *et al.* (1994). *E. coli* was transformed by electroporation with a Bio-Rad (Hercules, CA) gene pulser set at 25 μF and 2.46 kV (Sambrook *et al.* 1989). Plasmids were purified from *E. coli* and *M. maripaludis* using the Wizard Plus Miniprep DNA purification system (Promega) following the manufacturer's instructions. DNA was electrophoresed on 0.8% agarose gels as described by Sambrook *et al.* (1989).

Base analog selection and isolation of mutants: Auxotrophic mutants of *M. maripaludis* were enriched by a modification of the nucleobase selection of Ladapo and Whitman (1990). Enrichment medium was prepared by adding 50 mg of 8-aza-hypoxanthine and 10 mg of 6-azauracil to sterile culture tubes. The culture tubes were incubated for 1 day in the anaerobic chamber before 5 ml of sterile McN medium was added. After stoppering, the gas atmosphere was exchanged with $H_2 + CO_2$. The frozen stock culture of the transformants (0.2 ml) was inoculated into 5 ml of McAC$^+$ medium and incubated overnight at 37°. When the A_{600} was 0.7, 0.2 ml was inoculated into the enrichment medium. After incubation for 2 days at 37°, the enrichment culture was centrifuged at 2000 × *g* for 20 min at room temperature, and the cell pellet was resuspended in 5 ml of McN medium. The cells were washed one additional time and resuspended in McAC$^+$ medium. The culture was then incubated at 37° until the A_{600} was >0.5, and 0.2 ml was inoculated into enrichment medium. After incubation for 2 days, the cells were washed in McN medium and resuspended in McC$^+$ medium as described above. Culture (0.2 ml) was then plated on McC$^+$ plus 2.5 μg ml^{-1} of puromycin. Plates were incubated for 7 days, and isolated colonies were replica plated on McN, McA, McC, and McAC$^+$ media.

Southern hybridizations: Genomic DNA was purified by the miniprep procedure of Wilson (1994). Genomic DNA (14 μg) was treated with 72 units of *Eco*RI for 48 hr. After electrophoresis of 6 μg, the DNA was transferred to positively charged nylon membranes (Boehringer Mannheim, Indianapolis) following the manufacturer's protocol. The probe, pWDK117, was labeled with Dig-dUTP according to the protocol of the Genius labeling kit (Boehringer Mannheim). Prehybridization and hybridization were performed at 65° for 12 and 24 hr, respectively. Hybridization was visualized with CSPD Ready-to-Use Chemiluminescent Substrate (Boehringer Mannheim).

Isolation and sequencing of plasmids from transformants: To obtain the plasmids from the *M. maripaludis* mutants, a 5-ml culture in McC$^+$ plus 2.5 μg ml^{-1} puromycin was harvested by centrifugation at 2000 × *g* for 20 min at room temperature. The cells were resuspended in 200 μl of a solution containing 50 mm Tris chloride, pH 7.5, 10 mm sodium EDTA, and 100 μg ml^{-1} DNase-free RNase A. The suspension was then treated using the Wizard Plus Miniprep DNA Purification System (Promega) according to the manufacturer's instructions. A portion of the purified DNA (15 μl in deionized water) was dialyzed for 2 hr at room temperature. Dialysis was

performed by placing a drop of the suspension on the surface of a 0.025-μm filter disk (2.5 cm diameter, Type VS; Millipore, Bedford, MA), and the filter disk was floated on the surface of 20 ml of deionized water in a petri dish. After dialysis, *E. coli* SURE cells were electroporated with 1–5 μl of the DNA suspension.

The plasmids were then isolated from the *E. coli* transformants as described above. The genomic inserts were then sequenced using the primers 5′-AGGCACCCCAGGCTTTA CAC and 5′-GCGTTTTTTATTACCTACTA, which were complementary to the flanking regions of pMEB.2. Sequencing was performed on an Applied Biosystems (Foster City, CA) model 373 automated sequencer at the Molecular Genetics Instrumentation facility at the University of Georgia. Sequences were analyzed with the FASTA and BLAST programs available through GCG (University of Wisconsin) and at the TIGR website (http://www.tigr.org).

Construction of pWDK117-11 and pWDK117-P: An internal fragment of pWDK117 was subcloned. For pWDK117-11, pWDK117 was digested with *Bam*HI and *Bgl*II, and the 682 internal fragment was gel purified and ligated into the *Bgl*II site of pWGL11 (Gardner and Whitman 1999). In the resulting plasmid, the direction of transcription of the gene fragment was in the opposite direction of the *pac* cassette. For pWDK117-P, pWDK117 was digested with *Bgl*II, and the 316-bp product containing the 5′-end of the open reading frame (ORF) was gel purified and ligated into the *Bgl*II site of pIJA02. This plasmid was similar to pWLG14 and contained the *M. voltae* histone promoter PhmvA immediately upstream of the *Bgl*II site (Gardner and Whitman 1999; I. J. Anderson and W. B. Whitman, unpublished data).

Accession numbers: The GenBank accession numbers for the *M. maripaludis* DNA cloned in pWDK101, pWDK103, pWDK104, pWDK106, pWDK107, and pWDK117 were AF147208, AF146562, AF146563, AF146564, AF146565, and AF146566, respectively.

RESULTS

Library construction: A plasmid library for random mutagenesis of *M. maripaludis* was constructed by blunt end ligation of sonicated genomic DNA into the *Eco*RV site of pMEB.2. The initial library was electroporated into *E. coli*, where 3×10^4 transformants were found. However, of 28 transformants screened, only 21 possessed cloned genomic DNA. Thus, the number of clones in the library was estimated as 2.3×10^4. The size distribution of 20 of these clones was as follows: <0.5 kb, 4; 0.5–1.0 kb, 4; 1.0–1.5 kb, 9; 1.5–2.0 kb, 2; >2.0 kb, 1. Upon transformation of *M. maripaludis*, 3.3×10^4 transformants were found. Assuming a genome size of 2000 kb and average clone size of 1 kb, a library of 9200 transformants would have a 99% probability of containing any gene of interest. By this criterion, the library was large enough to provide insertions throughout the genome. However, replica plating of 700 transformants of *M. maripaludis* failed to identify conditional mutants that grew poorly in minimal medium (McN). The failure to identify large numbers of conditional mutants could have resulted from a number of effects. If the cloned DNA were too large, it would not be internal to an operon and integration of the plasmid would not disrupt function. Although the average size of operons in methanococci is not known, in *M. jannaschii* about 50% of the ORFs are >600 bp. If the average transcriptional unit is only two ORFs, only a small percentage of the clones in the library would be internal. This problem may have been exacerbated by amplification in *M. maripaludis*, which would enrich for transformants from large clones that have a higher recombination frequency. In addition, auxotrophs that grew poorly in the complex medium may have been lost during the amplification in *M. maripaludis*. Last, the amplification of the library in *E. coli* could have further reduced its variability. In any case, the reason for the low number of conditional mutants in the library was not explored further.

TABLE 1

Mutants of *M. maripaludis* isolated by random insertional mutagenesis

	Growth characteristics[a]				
Mutant	McN	McA	McC	McAC$^+$	Plasmid isolated[b]
JJ101	±	±	±	+	pWDK101
JJ102	±	±	±	+	pWDK102
JJ103	±	±	±	+	pWDK103
JJ104	±	+	+	+	pWDK104
JJ105	±	±	±	+	pWDK105
JJ106	±	±	±	+	pWDK106
JJ107	±	±	±	±	pWDK107
JJ108	±	±	+	+	None[c]
JJ117	−	+	+	+	PWDK117

[a] Growth of mutants on replica plates containing mineral (autotrophic) medium (McN), mineral medium + acetate (McA), mineral medium + yeast extract (McC), and rich, complex medium (McAC$^+$). −, no growth; ±, poor growth, +, good growth.

[b] Plasmid isolate from the mutant by electroporation into *E. coli*.

[c] No plasmid was isolated.

Isolation and characterization of JJ104 and other auxotrophs: Because of the low frequency of conditional mutants, auxotrophs were enriched by base analog selection. In the first experiment, eight potential auxotrophs were identified among 100 transformants (Table 1). These auxotrophs, JJ101–JJ108, all grew poorly in mineral medium. For one of them, JJ104, good growth was restored by the addition of acetate. JJ108 required yeast extract for good growth. Five of the remaining six auxotrophs required McAC$^+$, a highly enriched medium containing acetate as well as amino acids and vitamins, for good growth; the sixth auxotroph, JJ107, grew poorly even in rich medium.

If the phenotypes were caused by integration of plasmids by homologous recombination, then revertants should occur at a low frequency due to recombination of the plasmids out of the genome. Because the plasmids lack a methanococcal origin of replication, they would normally be lost from the culture. Even though their

abundance was expected to be low, it was possible to isolate these plasmids by electroporation of plasmid preparations from the *M. maripaludis* auxotrophs into *E. coli*. For seven of the eight mutants, plasmids were isolated in this fashion (Table 1). In all cases examined by restriction endonuclease digestions, the plasmids isolated from one mutant either contained genomic inserts of the same size or a genomic insert was absent and the digestion pattern was the same as that of the parent pMEB.2. These latter plasmids were discarded. Sequencing of the ends of the inserts indicated that pWDK101, pWDK102, and pWDK105 were identical. Presumably, the strains JJ101, JJ102, and JJ105 that yielded these plasmids represented multiple isolations of the same mutant during the enrichment and were not formed by independent mutations. Nevertheless, this result demonstrated that the same plasmid could be recovered from multiple clones of a mutant. Even after several attempts, it was not possible to isolate a plasmid from JJ108.

Sequencing of the genomic inserts in the plasmids identified open reading frames homologous to ORFs in the genomic sequences of *M. jannaschii* and *Methanobacterium thermoautotrophicum* (Figure 1; Bult *et al.* 1996; Smith *et al.* 1997). pWDK104 encoded components homologous to ABC transporters, including the 3′-end of an integral membrane protein and the 5′-end of an ATP-binding subunit. The partial sequence of the integral membrane protein component had high similarity to the *M. jannaschii* ORF annotated as a sulfate/thiosulfate transporter and contained the conserved EAA loop region. The second partial ORF had high similarity to the *M. jannaschii* ORF annotated as a sulfate permease and contained the Walker motif A (Boos and Lucht 1996). pWDK101 contained three small ORFs of 75–110 amino acid residues. None of these ORFs displayed significant sequence similarity to ORFs in *M. jannaschii* or *M. thermoautotrophicum*. Because of their small size and the absence of homologs, it was not clear that these ORFs were in fact expressed. pWDK103 contained an ORF homologous to the 3′- end of a hypothetical membrane-spanning protein common to *M. jannaschii* and *M. thermoautotrophicum*, an ORF with no known homologs, as well as an ORF homologous to the thiamine biosynthetic protein *thiI*. However, when compared to the *M. jannaschii thiI*, the *M. maripaludis* ORF was missing 101 amino acid residues from the N terminus. Examination of other reading frames did not produce evidence for a frame shift in the sequence. Therefore, this gene appeared to be truncated or possibly split in *M. maripaludis*. pWDK106 contained a pair of ORFs with homology to the hypothetical proteins in *M. jannaschii* MJ0835 and MJ0835.1. This plasmid and pWDK104 were the only cases in which the gene order in *M. jannaschii* was conserved in *M. maripaludis*. Last, pWDK107 contained two divergently translated ORFs. The large ORF had homology to an ORF annotated as an endo-1,4-β-glucanase in both *M. jannaschii* and *M. thermoautotrophicum*. The small ORF was homologous to the transketolase of *M. jannaschii*. *M. thermoautotrophicum* does not possess this enzyme, and no homolog was observed (Smith *et al.* 1997).

Because the methanococcal genome is AT rich, it is difficult to recognize transcriptional start sites on the basis of sequence information alone. Nevertheless, on the basis of the organization of the ORFs, pWDK103, pWDK104, and pWDK106 may well have encoded regions internal to an operon, as expected from the method of mutagenesis. However, pWDK107, which encoded two divergently translated ORFs, was likely to overlap the transcriptional units, and a simple insertion of the vector would not have been expected to inactivate these genes. In this case, the phenotype could have resulted from more complex insertional events.

Southern hybridizations of the genomic DNA of these mutants with pMEB.2 as the probe indicated that these plasmids had integrated as tandem repeats (data not shown). However, it was not determined whether the repeats represented the initial product of the recombination or were subsequently formed during the enrichment and subculturing of the mutants. In other experiments (see below), tandem repeats appeared to represent about 10% of the products formed by recombination of similar plasmids into the genome. Presumably, the high proportion of tandem repeats obtained in these experiments could have resulted from the initial selection of the methanococcal library in puromycin-containing medium.

Phenotype of JJ104: In the initial screening by replica plating, growth of JJ104 was stimulated by acetate. Similarly, growth was also stimulated by yeast extract, which contains acetate in addition to amino acids and other potential nutrients. To confirm that the phenotype was due to insertion of pWDK104, the wild-type strain JJ1 was transformed with pWDK104 to form JJ104-1. Like the original mutant, growth of JJ104-1 was stimulated by acetate (Figure 2), confirming that the insertion of pWDK104 was sufficient to produce the original mutation. In addition, growth of JJ104-1 was stimulated by 0.2% (w/v) Casamino acids (data not shown). The ability of Casamino acids to substitute for acetate suggested that the growth stimulation was due to a general sparing of stress by organic carbon sources and not a specific requirement for acetate (Ladapo and Whitman 1990).

Sulfide is abundant in the habitats of methanococci and can serve as a sole sulfur source, and most mesophilic methanococci do not have a nutritional requirement for sulfate and other sulfur oxyanions (Whitman 1989). Thus, even though some methanococci can assimilate sulfur oxyanions like thiosulfate (Daniels *et al.* 1986), a mutation in a sulfur oxyanion transporter would not be expected to have a phenotype in sulfide-containing medium. Therefore, in spite of its assignment, the ABC transporter homolog encoded by

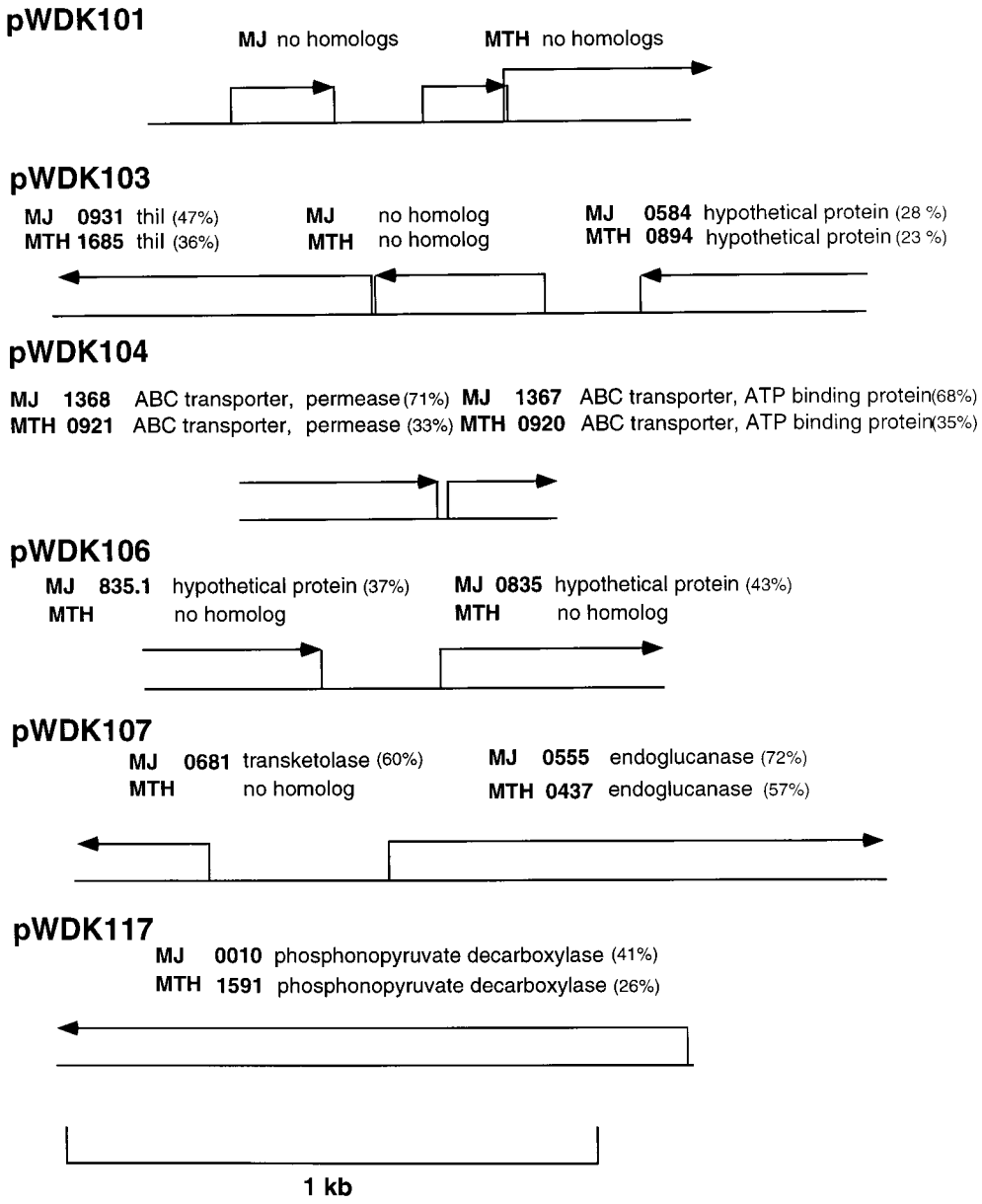

Figure 1.—Sequences of *M. maripaludis* genomic DNA cloned on plasmids isolated from the transformants. The cloned DNA was inserted at the *Eco*RV site adjacent to the *pac* cassette on pMEB.2. In the orientation shown, the *pac* cassette would be on the right. The direction of translation of the ORFs is indicated by arrows. Homologs in the genomic sequences of *M. jannaschii* (MJ) and *Methanobacterium thermoautotrophicum* (MTH) are indicated immediately above the ORFs, and the percentage sequence identity is indicated in parentheses. The 5′- and 3′-ends of the putative genes are indicated by vertical lines from the arrow. The alignment to the *M. jannaschii* homolog was used to choose between alternative start codons suggested by the sequence analysis. When no homolog was found, the ends of the putative genes were taken to be the putative translational start (ATG, GTG, or TTG codons) and termination sites.

pWDK104 was probably involved in uptake of some nutrient other than sulfur oxyanions. In support of this hypothesis, growth of JJ104-1 was stimulated by the addition of a mixture of molybdate, tungstate, and selenite (Figure 2). For the wild-type JJ1, a nutritional requirement for these oxyanions was apparent only after the second transfer in medium without added oxyanions (data not shown). Presumably, oxyanions contaminating the medium supported the nutritional requirements of the wild type during the first transfer. Thus, both the wild type and JJ104-1 required these oxyanions for growth, but JJ104-1 appeared to require higher concentrations. The addition of selenite (10 μm) by itself was also stimulatory, although it was not as effective as the mixture of three oxyanions (data not shown). The addition of tungstate (10 μm) by itself was inhibitory. This result would be expected if uptake of oxyanions in the mutant was performed by a nonspecific transporter and high levels of tungstate inhibited uptake of the other essential oxyanions.

Isolation of JJ117: In a second round of enrichment with base analogs, four additional auxotrophs were identified. One of these, JJ117, had an absolute requirement of acetate for good growth and was characterized further (Table 1). Upon electroporation of a plasmid preparation from JJ117 into *E. coli*, two transformants were found. Upon *Eco*RI digestion, the plasmids in both transformants appeared identical. The cloned genomic DNA of one, pWDK117, was sequenced, and a large ORF was identified (Figure 1). This ORF was homologous to two *M. jannaschii* ORFs, MJ0010 and MJ1612 (Bult *et al.* 1996). The *M. maripaludis* ORF also appeared to be truncated and missing the 27 and 6 C-terminal amino acids of MJ0010 and MJ1612, respectively. Moreover,

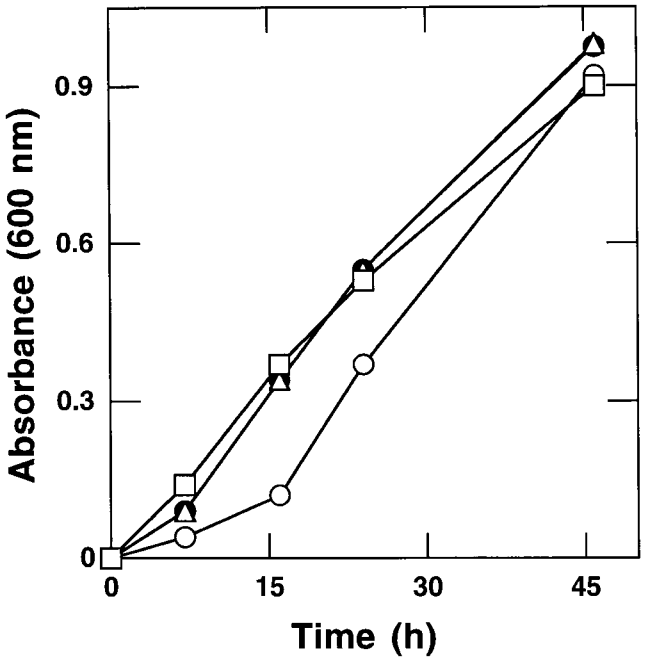

Figure 2.—Stimulation of growth of the acetate auxotroph JJ104-1 by acetate and the oxyanions molybdate, tungstate, and selenite. Growth of JJ104-1 in McN medium (○), in McN plus 10 mm acetate (●), and in McN plus 10 μm each of sodium molybdate, sodium tungstate, and sodium selenite (△). Growth of the wild-type JJ1 in McN medium without additions (□). Growth of the wild type in the presence of acetate or the oxyanions was identical to growth in McN medium without additions (data not shown). The inoculum was 8×10^6 cells or 4% (v/v) that had been grown in McAC$^+$ and washed twice in McN medium.

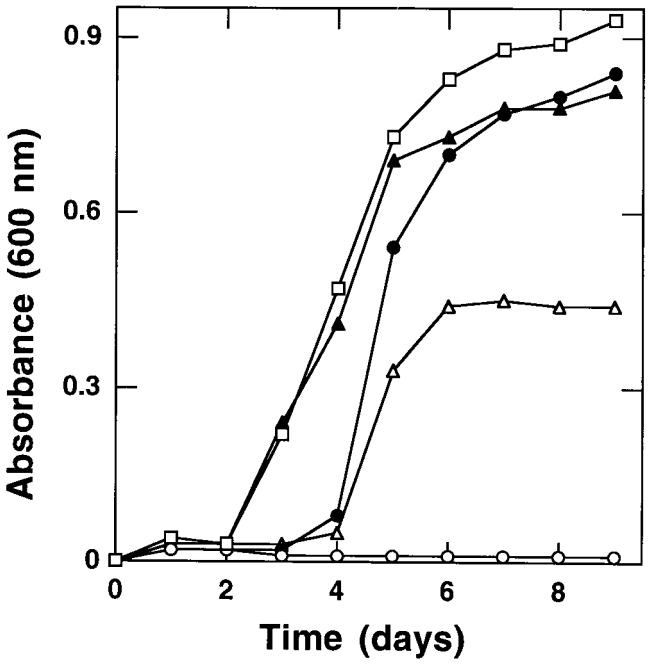

Figure 3.—Stimulation of growth of the acetate auxotroph JJ117 by acetate and vitamins. (○) Growth of JJ117 in McN medium; (●) growth in McN plus 10 mm acetate; (△) growth in McN plus a mixture of water-soluble vitamins; and (▲) growth in McN plus both acetate and vitamins; (□) growth of the wild type in McN medium. The inoculum was 6×10^3 cells that had been grown in McAC$^+$ medium.

the amino acid similarity was only 40 and 27% with MJ0010 and MJ1612, respectively, or much lower than observed for likely orthologs between *M. jannaschii* and *M. maripaludis.* Although MJ0010 and MJ1612 were both homologs of a Streptomyces gene (Bult *et al.* 1996), the functional assignment of this gene has been called into question. It was originally believed to encode phosphonopyruvate decarboxylase (Nakashita *et al.* 1997). This enzyme catalyzes the formation of phosphonoacetaldehyde from phosphonopyruvate and is a required early step in antibiotic biosynthesis. However, subsequent authors pointed out that the gene lacks a thiamine pyrophosphate binding motif and is unlikely to be a decarboxylase (Schwartz *et al.* 1998). The methanococcal ORFs also do not possess thiamine pyrophosphate binding motifs, and their functions are not known.

Phenotype of JJ117: In the initial screening, JJ117 failed to grow on plates in the absence of acetate. This phenotype was confirmed in broth cultures. Washed cells or very high dilutions of cultures failed to grow in mineral medium even after 10 days (Figure 3 and data not shown). However, in the presence of acetate, growth was restored. Moreover, the addition of Casamino acids or a mixture of branched-chain amino acids failed to support growth, suggesting that the nutritional requirement for acetate was specific and not due to a general sparing of energy metabolism by organic carbon sources. Surprisingly, a mixture of water soluble vitamins also supported growth (Figure 3). Of the vitamins, only cobalamin (1 μg liter^{-1}) supported growth, and thiamine (50 μg liter^{-1}), biotin (20 μg liter^{-1}), paraaminobenzoic acid (50 μg liter^{-1}), folic acid (20 μg liter^{-1}), riboflavin (50 μg liter^{-1}), nicotinic acid (50 μg liter^{-1}), pyridoxine HCl (100 μg liter^{-1}), and dl-calcium pantothenate (50 μg liter^{-1}) had no effect (data not shown). Good growth was also obtained with 80 μg liter^{-1} or 0.06 μm cobalamin, and no further stimulation was observed with concentrations as high as 7.5 μm (data not shown). Structural components of cobamides including cobinamide (0.8 μm), 5′,6′-dimethylbenzimidazole (34 μm), 2′-hydroxybenzimidazole (34 μm), 5′-methylbenzimidazole (34 μm), adenine (37 μm), guanine (37 μm), and 2-aminopropanol (1% v/v) did not support growth of JJ17 and did not inhibit growth of the wild-type JJ1 (data not shown). In methanococci, the natural cobamide contains 5′-hydroxybenzimidazole and not 5′,6′-dimethylbenzimidazole as found in cobalamin (Gorris and van der Drift 1994). Because 5′-hydroxybenzimidazole was not commercially available, a wide range of nucleosides and potential axial ligands were tested on the assumption that at least some of them could be assimilated. The failure of the components of cobalamin to replace the nutritional require-

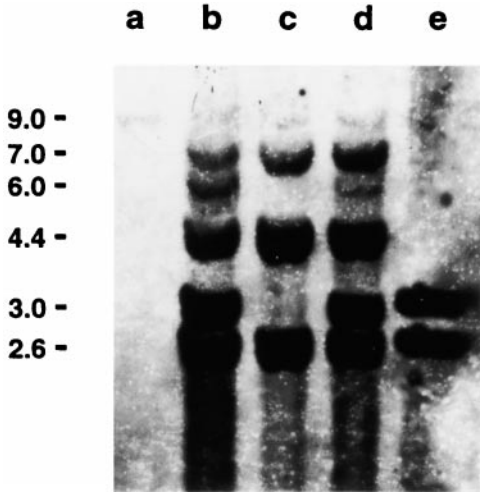

Figure 4.—Southern hybridization of pWDK117 to genomic DNA from *M. maripaludis* strains JJ1, JJ117, JJ118, and JJ119. DNA was treated with *Eco*RI prior to electrophoresis. The DNA was from (a) the wild-type strain JJ1, (b) the original auxotroph JJ117, (c) the transformant JJ119 with normal growth properties, (d) the transformant JJ118 that was auxotrophic, and (e) the plasmid pWDK117. The molecular weights of the major bands are indicated in the left margin.

ment in JJ117 suggested that the mutation in JJ117 did not affect biosynthesis of the cobinamide, benzimidazole, or the aminopropanol linker. Thus, the phenotype could result from an inability to assemble these components or by an indirect effect on cobamide metabolism.

Genotype of JJ117: To determine whether the phenotype of JJ117 was caused by insertion of pWDK117, the wild-type JJ1 was transformed with the plasmid. While 2 out of 17 transformants were unable to grow in mineral medium, the remainder grew normally. One of the auxotrophs, JJ118, was examined by Southern hybridization (Figure 4). In this experiment, the chromosomal DNA was digested with *Eco*RI. Hybridization with pWDK117 to itself produced two bands, representing the 2.6- and 3.0-kb *Eco*RI fragments. This plasmid hybridized weakly to a 9-kb fragment in the genomic DNA of the wild type. Presumably, this band contained the wild-type gene that had been cloned on the insert. The genomic DNA of JJ117 and JJ118 contained 2.6-, 3.0-, 4.4-, and 7.0-kb fragments that hybridized to pWDK117. In addition, a 6-kb fragment was observed in JJ117, which may have represented an incomplete digestion product. This pattern would be expected if pWDK117 integrated into the genome as tandem copies. In contrast, a transformant with normal growth properties, JJ119, contained only the 2.6-, 4.4-, and 7.0-kb *Eco*RI fragments. This pattern was expected if only a single copy of the plasmid had integrated. Thus, the auxotrophic phenotype appeared to be associated with tandem insertions of the plasmid.

Because single insertions of the pWDK117 did not produce the auxotrophic phenotype, it seemed unlikely that the phenotype was due to disruption of the large ORF. This hypothesis was supported by three additional observations. First, pWDK117 probably contained most of the ORF, and based upon its alignment with MJ0010 it was missing only 20–80 bp of the 3′- end. After homologous recombination, transformants with a single insertion such as JJ119 would be expected to express a nearly full-length ORF, which might retain activity and support normal growth. Second, a portion of the large ORF representing amino acid residues 81–309 was subcloned to produce a plasmid pWDK117-11. Transformation with pWDK117-11 should further truncate the large ORF from 403 to 309 amino acids. Of 47 transformants tested, none were auxotrophic. Third, it was also possible that the large ORF was essential for growth even in the presence of acetate. In this model, the truncated ORF would have had partial activity that only allowed for growth in the presence of acetate. Insertions which completely inactivated the gene would be lethal unless other more complicated genetic events also occurred, such as tandem insertions of the plasmid. If this were the case, the transformation efficiency of pWDK117-11 would be expected to be lower than that observed for pWDK104, which contains a genomic DNA fragment of nearly the same size as pWDK117-11. However, the transformation efficiencies of the two plasmids were nearly the same, indicating that the large ORF was not essential (data not shown). For these reasons, the phenotype of JJ117 did not appear to result from disruption of the large ORF.

It was also possible that the phenotype of JJ117 could be due to overexpression of the large ORF. In this scenario, the large ORF might be a cobamide-binding protein whose overexpression would titrate cobamides out of enzymes in methanogenesis and other essential functions. Acetate might spare this defect if biosynthesis of the cobamide Fe/S protein in the acetyl-CoA synthase complex represented a major pool of intracellular cobamide. To test this hypothesis, the wild-type strain was transformed with an integration expression vector, pWDK117-P. This plasmid contained the moderately strong histone promoter from *M. voltae*, P_{hmvA}, and 316 bp of the 5′-end of the large ORF. Upon integration, it was expected to place the genomic copy of the large ORF under control of P_{hmvA} and to cause overexpression of the gene product (Gardner and Whitman 1999). Of the five transformants examined, all grew well in minimal medium, and none were auxotrophic for acetate or cobalamin. In addition, examination of the sequence of the large ORF failed to identify a cobalamin-binding motif (Marsh and Holloway 1992; Chen and Marsh 1997). Thus, these experiments failed to provide evidence in support of this model.

DISCUSSION

Transposon mutagenesis is a valuable technique that has been applied only sporadically to the archaea (Tum-

bula and Whitman 1999). For instance, pMudpur, which contained the Mu transposon and the *pac* cassette for puromycin selection in methanococci, was created to mutagenize cloned methanococcal *nif* DNA (Blank *et al.* 1995). Upon transformation of the cloned DNA into *M. maripaludis*, transposon insertion was achieved by selecting for puromycin resistance.

An alternative to transposon mutagenesis is to disrupt genes by transformation with a nonreplicative plasmid containing randomly cloned fragments of genomic DNA (Larson and Hersheberger 1990; Law *et al.* 1995; Leloup *et al.* 1997). Campbell-like integration events insert the plasmid via homologous recombination at sites throughout the genome. The plasmid is inserted between two identical copies of the original cloned DNA. For this general strategy to be applied to the methanococci, it is necessary to obtain large numbers of random insertions throughout the genome, to recover the plasmid after isolation of specific mutants, and to confirm the phenotype after transforming the wild type with the plasmids isolated from specific mutants. For *M. maripaludis*, it has been possible to perform each of these steps during the isolation of acetate auxotrophs. Therefore, this method may have general utility in methanococcal genetics.

The first mutant, JJ104, was a leaky acetate auxotroph whose growth was also stimulated by amino acids and the oxyanions molybdate, tungstate, and selenite. Southern hybridization indicated that it contained tandem insertions of the vector into a homolog of an ABC transporter. This family of transporters has been characterized in the archaea by genomic sequencing (Bult *et al.* 1996; Jovell *et al.* 1996), and a maltose transporter from *Thermococcus litoralis* has been studied biochemically (Xavier *et al.* 1996; Horlacher *et al.* 1998). JJ104 is the first mutant isolated in archaea that disrupted their function *in vivo.* Although the transport function was not measured directly, the phenotype is consistent with a role in molybdate, tungstate, and selenite uptake. These oxyanions are required for biosynthesis of the active forms of the pterin coenzymes of the formylmethanofuran and formate dehydrogenases, the selenium hydrogenases, selenium-containing tRNAs, and probably other undiscovered macromolecules (see Thauer *et al.* 1993; Sorgenfrei *et al.* 1997; Wilting *et al.* 1997). Both amino acids and acetate can be major carbon sources for the facultative autotroph *M. maripaludis* (Whitman *et al.* 1987) and might be expected to stimulate when growth is limited for these essential oxyanions.

The second acetate auxotroph, JJ117, had an absolute requirement for either acetate or cobalamin. However, this phenotype probably did not result from simple inactivation of a gene in *M. maripaludis.* First, the original mutant contained tandem repeats of the vector and the cloned gene. Tandem repeats of insertion vectors are relatively common and have been observed before in methanococci (Gernhardt *et al.* 1990; Sandbeck and Leigh 1991). However, transformants of pWDK117 containing a single insert were not auxotrophic. When transformed with a plasmid pWDK117-11 containing smaller, internal fragments of the gene, auxotrophs were again not found. These experiments argue strongly against insertional inactivation causing the phenotype. Even though it was not possible to establish a causal link between the genotype and phenotype in JJ117, these experiments describe a new genetic locus important in acetate and cobamide metabolism in *M. maripaludis.*

The authors are grateful to Jae Pil Yu, Debra Tumbula, and Warren Gardner for helpful discussions and to Warren Gardner for providing pWLG11. This work was supported by grant DE-FG02-97ER20269 from the U.S. Department of Energy Division of Energy Biosciences.

LITERATURE CITED

Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese and R. S. Wolfe, 1979 Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43:** 260–296.

Berghofer, Y., and A. Klein, 1995 Insertional mutations in the hydrogenase *vhc* and *frc* operons encoding selenium-free hydrogenases in *Methanococcus voltae.* Appl. Environ. Microbiol. **61:** 1770–1775.

Blank, C. E., P. S. Kessler and J. A. Leigh, 1995 Genetics in methanogens: transposon insertion mutagenesis of a *Methanococcus maripaludis nifH* gene. J. Bacteriol. **177:** 5773–5777.

Boos, W., and J. M. Lucht, 1996 Periplasmic binding protein-dependent ABC transporters, pp. 1175–1209 in *Escherichia coli and Salmonella: Cellular and Molecular Biology*, edited by F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low *et al.* American Society for Microbiology, Washington, DC.

Bult, C. J., O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann *et al.*, 1996 Complete genome sequence of the methanogenic archaeon, Methanococcus jannaschii. Science **273:** 1058–1073.

Chen, H.-P., and E. N. G. Marsh, 1997 How enzymes control the reactivity of adenosylcobalamin: effect on coenzyme binding and catalysis of mutations in the conserved histidine-aspartate pair of glutamate mutase. Biochemistry **36:** 7884–7889.

Daniels, L., N. Belay and B. S. Rajagopal, 1986 Assimilatory reduction of sulfate and sulfite by methanogenic bacteria. Appl. Environ. Microbiol. **51:** 703–709.

Gardner, W. L., and W. B. Whitman, 1999 Expression vectors for *Methanococcus maripaludis*: overexpression of acetohydroxyacid synthase and β-galactosidase. Genetics **152:** 1439–1447.

Gernhardt, P., O. Possot, M. Foglino, L. Sibold and A. Klein, 1990 Construction of an integration vector for use in the archaebacterium *Methanococcus voltae* and expression of a eubacterial resistance gene. Mol. Gen. Genet. **221:** 273–279.

Gorris, L. G. M., and C Van Der Drift, 1994 Cofactor contents of methanogenic bacteria revisited. Biofactors **4:** 139–145.

Horlacher, R., K. B. Xavier, H. Santos, J. Diruggiero, M. Kossman *et al.*, 1998 Archaeal binding protein-dependent ABC transporter: molecular and biochemical analysis of the trehalose/maltose transport system of the hyperthermophilic archaeon *Thermococcus litoralis.* J. Bacteriol. **180:** 680–689.

Jarrell, K. F., D. P. Bayley, V. Florian and A. Klein, 1996 Isolation and characterization of insertional mutations in flagellin genes in the archaeon *Methanococcus voltae.* Mol. Microbiol. **20:** 657–666.

Jones, W. J., M. J. B. Paynter and R. Gupta, 1983 Characterization of *Methanococcus maripaludis* sp. nov., a new methanogen isolated from salt marsh sediment. Arch. Microbiol. **135:** 91–97.

Jovell, R. J., A. J. L. Macario and E. Conway De Macario, 1996 ABC transporters in Archaea: two genes encoding homologs of the nucleotide-binding components in the methanogen *Methanosarcina mazei* S-6. Gene **174:** 281–284.

Ladapo, J., and W. B. Whitman, 1990 Method for isolation of auxo-

trophs in the methanogenic archaebacteria: role of the acetyl-CoA pathway of autotrophic CO_2 fixation in *Methanococcus maripaludis*. Proc. Natl. Acad. Sci. USA **87:** 5598–5602.

Larson, J. L., and C. L. Hershberger, 1990 Insertion of plasmids into the chromosome of *Streptomyces griseofuscus*. Plasmid **23:** 252–256.

Law, J., G. Buist, A. Haandrikman, J. Kok, G. Venema *et al.*, 1995 A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. J. Bacteriol. **177:** 7011–7018.

Leloup, L., S. D. Ehrlich, M. Zagorec and F. Morel-Deville, 1997 Single-crossover integration in the *Lactobacillus sake* chromosome and insertional inactivation of the *ptsI* and *lacL* genes. Appl. Environ. Microbiol. **63:** 2117–2123.

Marsh, E. N. G., and D. E. Holloway, 1992 Cloning and sequencing of glutamate mutase component S from *Clostridium tetanomorphum*. FEBS Lett. **310:** 167–170.

Nakashita, H., K. Watanabe, O. Hara, T. Hidaka and H. Seto, 1997 Studies on the biosynthesis of bialaphos. Biochemical mechanism of C-P bond formation: discovery of phosphonopyruvate decarboxylase which catalyzes the formation of phosphonoacetaldehyde from phosphonopyruvate. J. Antibiot. **50:** 212–219.

Saito, H., and K.-I. Miura, 1963 Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta **72:** 619–629.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sandbeck, K. A., and J. A. Leigh, 1991 Recovery of an integration shuttle vector from tandem repeats in *Methanococcus maripaludis*. Appl. Environ. Microbiol. **57:** 2762–2763.

Schwartz, D., J. Recktenwald, S. Pelzer, and W. Wohlleben, 1998 Isolation and characterization of the PEP-phosphomutase and the phosphonopyruvate decarboxylase genes from the phosphinothricin tripeptide producer *Streptomyces viridochromogenes* Tu494. FEMS Microbiol. Lett. **163:** 149–157.

Shieh, J., and W. B. Whitman, 1988 Autotrophic acetyl coenzyme A biosynthesis in *Methanococcus maripaludis*. J. Bacteriol. **170:** 3072–3079.

Smith, D. S., L. A. Doucette-Stamm, C. Deloughery, H. Lee, J. Dubois *et al.* 1997 Complete genome sequence of *Methanobacterium thermoautotrophicum* ΔH: functional analysis and comparative genomics. J. Bacteriol. **179:** 7135–7155.

Sorgenfrei, O., S. Muller, M. Pfeiffer, I. Sneizko, and A. Klein, 1997 The [NiFe] hydrogenases of *Methanococcus voltae*: genes, enzymes and regulation. Arch. Microbiol. **167:** 189–195.

Thauer, R. K., R. Hedderick and R. Fischer, 1993 Reactions and enzymes involved in methanogenesis from CO_2 and H_2, pp. 209–252 in *Methanogenesis: Ecology, Physiology, Biochemistry and Genetics*, edited by J. G. Ferry. Chapman & Hall, New York.

Tumbula, D. L., and W. B. Whitman, 1999 Genetics of *Methanococcus*: possibilities for functional genomics in archaea. Mol. Microbiol. (in press).

Tumbula, D. L., R. A. Makula and W. B. Whitman, 1994 Transformation of *Methanococcus maripaludis* and identification of a *Pst*I-like restriction system. FEMS Microbiol. Lett. **121:** 309–314.

Tumbula, D. L., T. L. Bowen, and W. B. Whitman, 1995a Growth of methanogens on solidified medium, pp. 49–55 in *Archaea: A Laboratory Manual*, edited by F. T. Robb, K. R. Sowers and H. J. Schreier. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Tumbula, D. L., J. Keswani, J. Shieh and W. B. Whitman, 1995b Long-term maintenance of methanogen stock cultures in glycerol, pp. 85–87 in *Archaea: A Laboratory Manual*, edited by F. T. Robb, K. R. Sowers and H. J. Schreier. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Whitman, W. B., 1989 Order II. Methanococcales, pp. 2185–2190 in *Bergey's Manual of Systematic Bacteriology*, Vol. 3, edited by S. T. Staley, M. P. Bryant, N. Pfennig and J. G. Holt, Williams & Wilkins, Baltimore.

Whitman, W. B., 1994 Autotrophic acetyl coenzyme A biosynthesis in methanogens, pp. 521–538 in *Acetogenesis*, edited by H. L. Drake, Chapman & Hall, New York.

Whitman, W. B., J. Shieh, S. Sohn, D. S. Caras and U. Premachandran, 1986 Isolation and characterization of 22 mesophilic methanococci. Syst. Appl. Microbiol. **7:** 235–240.

Whitman, W. B., S. Sohn, S. Kuk and R. Xing, 1987 Role of amino acids and vitamins in nutrition of mesophilic *Methanococcus* spp. Appl. Environ. Microbiol. **53:** 2373–2378.

Wilson, K., 1994 Preparation of genomic DNA from bacteria. Curr. Protocols Mol. Biol. **1:** unit 2.4.

Wilting, R., S. Schorling, B. C. Persson and A. Bock, 1997 Selenoprotein synthesis in archaea: identification of an mRNA element of *Methanococcus jannaschii* probably directing selenocysteine insertion. J. Mol. Biol. **266:** 637–641.

Xavier, K. B., L. O. Martins, R. Peist, M. Kossman, W. Boos *et al.*, 1996 High-affinity maltose/trehalose transport system in the hyperthermophilic archaeon *Thermococcus litoralis*. J. Bacteriol. **178:** 4773–4777.

Communicating editor: A. Klein