Expression Vectors for *Methanococcus maripaludis*: Overexpression of Acetohydroxyacid Synthase and β-Galactosidase

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ABSTRACT

A series of integrative and shuttle expression vectors was developed for use in *Methanococcus maripaludis*. The integrative expression vectors contained the *Methanococcus voltae* histone promoter and multiple cloning sites designed for efficient cloning of DNA. Upon transformation, they can be used to overexpress specific homologous genes in *M. maripaludis*. When tested with *ilvBN*, which encodes the large and small subunits of acetohydroxyacid synthase, transformants possessed specific activity 13-fold higher than that of the wild type. An expression shuttle vector, based on the cryptic plasmid pURB500 and the components of the integrative vector, was also developed for the expression of heterologous genes in *M. maripaludis*. The β-galactosidase gene from *Escherichia coli* was expressed to ~1% of the total cellular protein using this vector. During this work, the genes for the acetohydroxyacid synthase (*ilvBN*) and phosphoenolpyruvate synthase (*ppsA*) were sequenced from a *M. maripaludis* genomic library.

METHANOGENS are important catalysts in the global carbon cycle. These strictly anaerobic archaea are the source of most of the earth's atmospheric methane, a potent greenhouse gas, and they are responsible for processing 1–2% of the carbon fixed every year during photosynthesis (Reeburgh *et al.* 1993). Given the enormity of this biogeochemical process, it is not surprising that methanogens are found in many different types of environments, ranging from psychrophilic to hyperthermophilic conditions (for a review see Boone *et al.* 1993). In this regard, they are the most cosmopolitan archaea; they are abundant in extreme environments, such as deep-sea hydrothermal vents, as well as temperate environments, such as the gastrointestinal tracts of man and animals.

Methanococcus maripaludis strain JJ is typical of other hydrogenotrophic methanococci. It was isolated from the sediment of a salt water marsh and grows by the conversion of carbon dioxide and hydrogen gas or formate into methane (Jones *et al.* 1983a). Amino acids and acetate are assimilated, but they are not significantly metabolized, indicating that methanogenesis is the sole or major energy source (Whitman *et al.* 1987). An important characteristic of *M. maripaludis* is its relatively rapid doubling time of 2.3 hr at the optimal temperatures of 35–39° (Jones *et al.* 1983a). Thus, it is one of the fastest growing mesophilic methanogens.

M. maripaludis has also proven amenable to genetic analysis (Tumbula and Whitman 1999). Integrative and shuttle vectors have been developed for Methanococcus species (Gernhardt *et al.* 1990; Cohen-Kupiec *et al.* 1997; Tumbula *et al.* 1997). These vectors are efficiently transformed into *M. maripaludis* by a polyethylene glycol-based method (Tumbula *et al.* 1994). Like other methanogens, *M. maripaludis* contains many oxygen-sensitive enzymes with complex prosthetic groups. These enzymes are not frequently expressed in an active form in *Escherichia coli*. Thus, a genetic system in *M. maripaludis* might be suitable for the expression of genes encoding these types of enzymes. We report here the development of expression shuttle and integrative vectors for *M. maripaludis* that use the *M. voltae* histone promoter (P_{hmvA}).

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MATERIALS AND METHODS

Bacterial strains, media, and growth conditions: *M. maripaludis* JJ was obtained from W. J. Jones. The plasmids used in this study are listed in Table 1. *M. maripaludis* was grown at 37° on 275 kPa of H_2:CO_2 (80:20) in McN (mineral medium), McC (complex medium minus the vitamin solution), or McNA (McN plus 10 mm sodium acetate; Whitman *et al.* 1986). The sodium bicarbonate in the medium was reduced from 5 g liter^{-1} to 2 g liter^{-1} during growth at 100 kPa in 1-liter Wheaton bottles. Transformation of *M. maripaludis* with plasmid DNA was described previously (Tumbula *et al.* 1994). For selection of puromycin-resistant methanococci, a stock solution of 500 μg ml^{-1} puromycin dihydrochloride (Sigma, St. Louis) in distilled water was filter sterilized and added to the medium at a final concentration of 2.5 μg ml^{-1}. Strictly anaerobic techniques were used for medium preparation and cultivation.

The *E. coli* strains were grown at 37° on low-salt Luria-Bertani (LB) medium with the NaCl at 50% of the regular concentration (Maniatis *et al.* 1982). Antibiotic concentrations were 60 μg ml^{-1} for ampicillin and 50 μg ml^{-1} for kanamycin in both liquid and solid LB medium. β-Galactosidase was used as a blue/white screen on LB agar plates with isopropylthio-

TABLE 1

Plasmids used in this study[a]

Plasmids	Properties	Reference
Mipuid	Contains *M. voltae* histone promoter with *uidA*, ampicillin resistance (amp^r)	Beneke *et al.* (1995)
pBK-CMV	Amp^r	Stratagene
pGEM-T	Amp^r	Promega
pGEM-T + *lacZ*	*lacZ* with flanking *Nsi*I and *Bgl*II sites, amp^r	This work
pMEB.2	pUC derivative that contains puromycin resistance cassette (*pac*) for methanococci (pur^r)	Gernhardt *et al.* (1990)
pTer7	Contains *E. coli* wild-type *lacZ*, amp^r	Elliot Altman
pTLB30	Contains *M. aeolicus* acetohydroxyacid synthase genes (*ilvBN*), amp^r	Bowen *et al.* (1997)
pUC18	Amp^r	
pURB500	Cryptic plasmid of *M. maripaludis* C5	Tumbula *et al.* (1997)
pWLG1	Contains *M. maripaludis ilvBN* and *ppsA*, kanamycin resistance (kan^r)	This work
pWLG2	3.8-kbp deletion of pWLG1, kan^r	This work
pWLG3	pUC18 plus 3.8-kbp insert from pWLG1, amp^r	This work
pWLG11	pMEB.2 derivative with multiple cloning site, amp^r, pur^r	This work
pWLG12A	pWLG11 plus *M. voltae* histone promoter (P_{hmvA}) with *uidA*, amp^r, pur^r	This work
pWLG13	Integrative expression vector with *Nde*I site downstream of P_{hmvA}, amp^r, pur^r	This work
pWLG13 + 5′-*ilvB*	Contains *M. maripaludis ilvB* fragment	This work
pWLG14	Integrative expression vector with *Nsi*I site downstream of P_{hmvA}, amp^r, pur^r	This work
pWLG18	pWLG14 with *lacZ* for blue/white screen	This work
pWLG30	Expression shuttle vector for *M. maripaludis*, amp^r, pur^{r-}	This work
pWLG30 + *lacZ*	Contains *lacZ* for *E. coli* blue/white screen	This work
pZeRO-2	Requires *E. coli* Top 10, kan^r	Invitrogen

[a] Ampicillin (amp) and kanamycin (kan) are for selection in *E. coli*. Puromycin (pur) is for selection in *M. maripaludis*.

β-d-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) according to the method of Sambrook *et al.* (1989). Plasmids were transformed into *E. coli* strains by electroporation with a Gene Pulser (Bio-Rad, Richmond, CA) using the settings of 200 Ω, 2.5 kV, and 25 μF with cuvettes (0.2 cm gap width).

Growth rates of *E. coli* and *M. maripaludis* were measured at 600 nm with a spectrophotometer (Spectronic 20).

Plasmid purification from *M. maripaludis* and *E. coli*: Plasmid isolation from *M. maripaludis* was begun by centrifuging a 5-ml culture at 16,000 × *g* at 4° followed by resuspension of the cells in 100 μl of methanococcal medium. After resuspension, the Wizard miniprep kit (Promega, Madison, WI) minus the resuspension step was used for preparation of the plasmid DNA for transformation into *E. coli*. pURB500 was isolated from *M. maripaludis* C5 as described previously (Tumbula *et al.* 1994). Plasmids from *E. coli*, grown with selective conditions, were isolated with the Wizard miniprep kit.

Construction of *M. maripaludis* genomic library: *M. maripaludis* genomic DNA was isolated using a modified procedure from Saito and Miura (1963). *M. maripaludis* was grown in 150 ml of McN to A_{600} = 0.8. The cells were harvested by centrifugation at 7700 × *g* at 4° for 15 min in a JA-21 rotor (Beckman, Fullerton, CA) and washed once in 28 ml of sterile 0.4 m NaCl. The cell pellet was then resuspended in 3 ml of 0.15 m NaCl/0.1 m EDTA (pH 8.0). The cell suspension was frozen by plunging the centrifuge tube into an ethanol/dry ice bath. The frozen suspension was slowly thawed at 18°, at which time the cells lysed. The cell extract was then transferred to a Corex tube, and 5 ml of 50% phenol saturated with 0.15 m NaCl/0.1 m EDTA (pH 8.0) and 50% chloroform:isoamyl alcohol (24:1 v/v) were added. The solution was gently mixed by inversion before centrifugation at 8200 × *g* for 10 min. The red aqueous phase was extracted with another 5 ml of phenol/chloroform/isoamyl alcohol. Two volumes of cold 100% ethanol were then added to the aqueous phase, and the suspension was gently inverted before centrifugation at 4° for 15 min at 8200 × *g*. The supernatant was decanted, and the pellet was dried at room temperature. The pellet was resuspended in 100 μl of 15 mm NaCl/1.5 mm trisodium citrate (SSC) and stored at 4°. Additional SSC was added every 24 hr until the DNA was resuspended.

To make a genomic library for *M. maripaludis*, the genomic DNA (12 μg in 100 μl) was partially digested with 0.01 units of *Sau*3AI at 37° for 5 min. The reaction was terminated by the addition of phenol/chloroform/isoamyl alcohol, pH 8.0 (Ameresco). The resulting linear DNA fragments ranged from 1 to 6 kbp, as measured by gel electrophoresis. The DNA was precipitated upon the addition of one-half volume of 7.5 m ammonium acetate and two volumes of cold 100% ethanol followed by centrifugation. After the supernatant was decanted, the pellet was resuspended in 10 μl of distilled water. Five micrograms of digested DNA (5 μl) was ligated with 2 μl of Zap Express (Stratagene, La Jolla, CA) that was predigested with *Bam*HI in a 10-μl reaction. After the ligation, the mixture was packaged and amplified once in *E. coli* XLOLR

(Stratagene) before storage at −80° according to the company's directions.

Isolation and sequencing of the *M. maripaludis ilvBN*: The *M. maripaludis* genomic library was screened with a polymerase chain reaction (PCR) product of the acetohydroxyacid synthase (AHAS) large subunit gene (*ilvB*) from *M. aeolicus* using pTLB30 as the template. The PCR contained the primers 5′-GAACGGAGCAGAGGC-3′ and 5′-ACCAACCATACCAAGG GC-3′, 5 units *Taq* DNA polymerase, 1 mm dATP, 1 mm dGTP, 1 mm dCTP, 0.65 mm dTTP, and 0.35 mm digoxigenin-deoxyuridine-5′-triphosphate (DIG-dUTP) from Boehringer Mannheim (Indianapolis, IN). The annealing temperature was 48°, and the extension time was 2 min at 72°. This DIG-dUTP-labeled PCR product was used to screen plaque lifts from the library according to Boehringer Mannheim protocols. The hybridization temperature was 63°. The isolated phagemid was then converted to the plasmid form (pWLG1). *Sac*I was used to subclone a 3.8-kbp fragment of pWLG1. This fragment was ligated into a *Sac*I-digested pUC18, yielding pWLG3. The remaining portion of pWLG1 was religated together to form pWLG2. These three plasmids were sequenced by primer walking utilizing API sequencers at the Molecular Genetics Facility, University of Georgia. All oligonucleotides were made by Integrated DNA Technologies (Coralville, IA). Sequence analysis was performed with the GCG software package (Genetics Computer Group, Madison, WI). FRAMES was used to identify open reading frames (ORFs) using ATG, GTG, or TTG as the start codons. GAP was used to calculate the percentage identities between the methanococcal proteins. PILEUP was used to identify the truncated regions for the phosphoenopyruvate synthase gene (*ppsA*) and the AHAS small subunit gene (*ilvN*).

Plasmid construction: The integrative vectors developed in this study were based on pMEB.2 (Figure 1). To remove the α-complementation fragment of *lacZ*, pMEB.2 was digested with *Nde*I and *Sac*I, followed by mung bean nuclease treatment and blunt-end ligation (Figure 1). This deletion allowed a unique *Nde*I site to be introduced into the multiple cloning site (MCS). The MCS was added after digestion of the plasmid with *Eco*RV and *Eco*RI and dephosphorylation with CIAP (calf intestine alkaline phosphatase). The phosphorylated oligonucleotides, 5′-AATTCAAGCATCATATGAAGCATACGCGTCT TAAGAGATCTCATGAT-3′ and 5′-ATCATGAGATCTCTTAA GACGCGTATGCTTCATATGATGCTTG-3′, were annealed and ligated into the vector forming pWLG11. The *Eco*RI fragment of Mipuid, which contained the *M. voltae* histone promoter (P_{hmvA}) and ribosome binding site and the *E. coli* β-glucuronidase (*uidA*), was then cloned into the *Eco*RI site of pWLG11. Removal of *uidA* by *Nde*I digestion formed the *M. maripaludis* integrative expression vector pWLG13. To form pWLG13+5′-*ilvB*, the first 641 bp of the *M. maripaludis ilvB* were cloned into pWLG13. First, the 5′ end of *ilvB* was amplified by PCR using the primers 5′-AAAAAAACATATGAAAG GAGCAGAGGCTATGATG-3′ and 5′-AAAAAAAGATCTCCG CCTGCAATAATAACAGGTCTT-3′. These primers contained *Nde*I and *Bgl*II sites at the 5′ and 3′ ends, respectively. The PCR contained 2.5 units of *Pfu* DNA polymerase (Stratagene), and it was performed at an annealing temperature of 55° for 1 min and an extension time of 2 min at 72°. The PCR product was drop dialyzed against distilled water with VSWP membranes (Millipore, Bedford, MA) before digestion with *Nde*I and *Bgl*II for 4 hr. The enzymes in the reactions were removed using the Wizard clean-up kit (Promega).

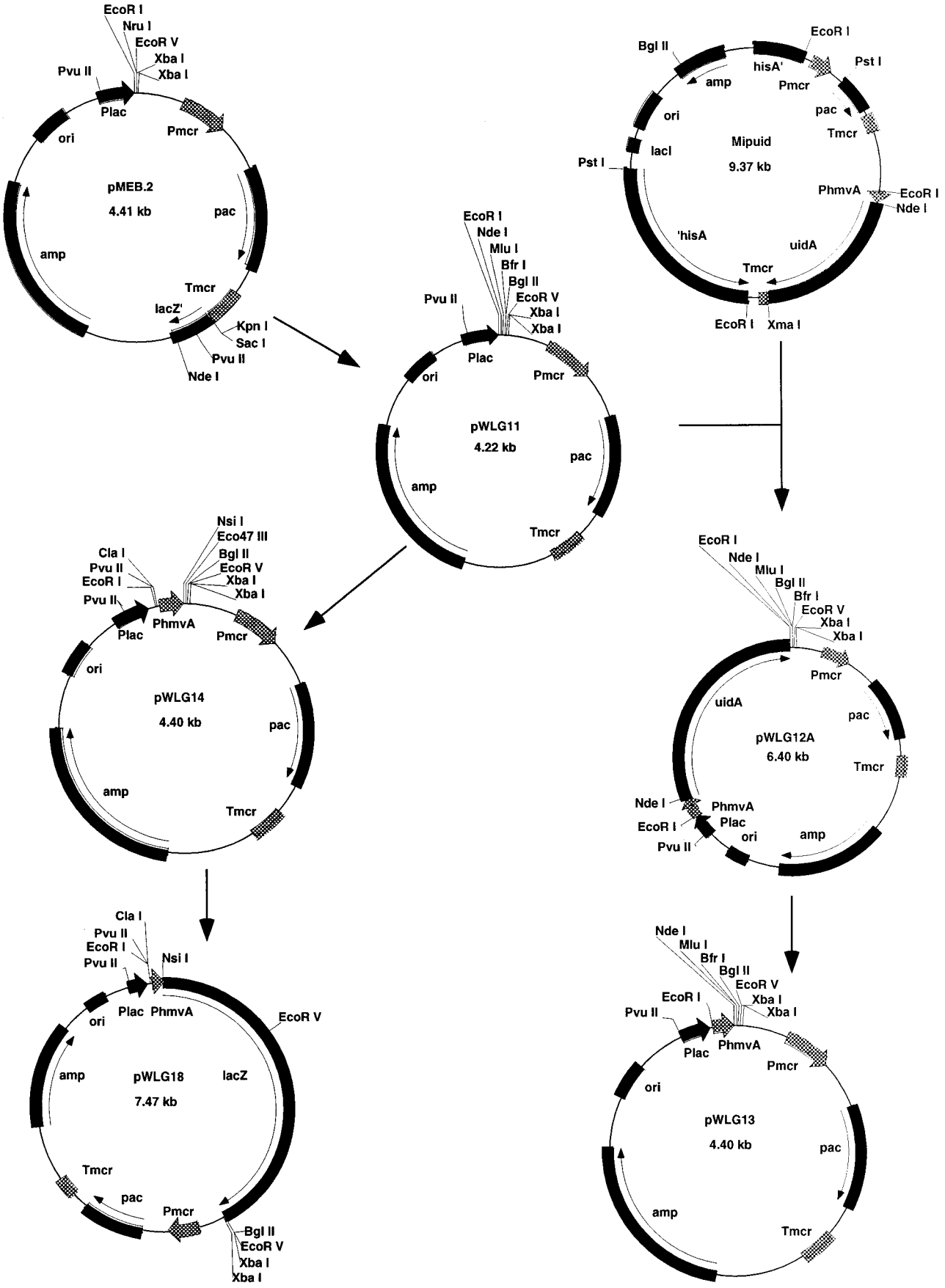
After the digestion of pWLG13 with *Nde*I and *Bgl*II, the PCR product was ligated in to form pWLG13+5′-*ilvB*. The sequence of the PCR product was confirmed by sequencing with the primers that flanked the insertion site, pMEB.2seq1, 5′-AGGCACCCCAGGCTTTACAC-3′ (5′ end), and pMEB.2-seq2, 5′-GCGTTTTTTATTACCTACTA-3′ (3′ end).

To replace the *Nde*I site near the histone promoter of pWLG13 with a *Nsi*I site, the promoter was PCR amplified using the primers 5′-ATCTGCGAATTCAGCTGATCGAT CAAAATATAACATAAATAACATAGGTTTAA-3′ (P_{hmvA}*Nsi*I1) and 5′-GAAGATCTTCAGCGCTAAACATGCATTTCACCTA TTAGTTATCTATAAAATTATA-3′ (P_{hmvA}*Nsi*I2). The first 12 nucleotides of P_{hmvA}*Nsi*I1 contained an *Eco*RI site and 5′ extension for *Eco*RI digestion. Restriction sites for *Pvu*II and *Cla*I were also added immediately upstream of the *M. voltae* histone promoter. P_{hmvA}*Nsi*I2 possessed a *Bgl*II site plus a two-nucleotide extension that allowed endonuclease digestion. The PCR annealing temperature was 43°, and the extension time was 1 min at 72°. The PCR product was digested with *Eco*RI and *Bgl*II for directed ligation into pWLG11, which had been digested with the same enzymes and treated with CIAP. The product, pWLG14, contained the *Nsi*I site as part of the start codon downstream of the histone promoter.

The *E. coli lacZ* gene was engineered by PCR for ligation into pWLG14. The PCR contained pTer7 as the template and the primers 5′-CCAATGCATGACCATGATTACGGATTCA CTGG-3′ and 5′-GAAGATCTTTCCTTACGCGAAATACGGG CAG-3′ to introduce flanking *Nsi*I and *Bgl*II sites. It was performed with 2.5 units of *Taq* DNA polymerase and an annealing temperature of 52.3° and an extension time of 3.75 min at 72°. The *lacZ* product was cloned into pGEM-T, yielding pGEM-T+*lacZ*. This vector was digested with *Nsi*I and *Bgl*II to release the *lacZ* fragment. After treatment of pWLG14 with *Nsi*I and *Bgl*II followed by CIAP, the *lacZ* fragment was ligated in to yield pWLG18. Upon electroporation into *E. coli* XL1-Blue MRF′ (Stratagene), pWLG18 was identified by β-galactosidase production on plates that contained X-gal and IPTG.

pWLG30, the expression shuttle vector, was constructed by ligation of a partial *Eco*RI digestion of pURB500 with a complete *Eco*RI digestion of pWLG14 (Figure 2). The ligation mixture was then transformed into *M. maripaludis*. The transformants were then inoculated into McC/puromycin broth to select for plasmids that contained both a methanococcal origin of replication from pURB500 and the *pac* cassette from pWLG14. Plasmids were then prepared from the broth culture of the transformants and electroporated into *E. coli* XL1-Blue MRF′. The location and orientation of pWLG14 within pURB500 was determined by digestion with *Sac*II and *Eco*O109I and was confirmed by sequencing. To form pWLG30 + *lacZ*, pWLG30 was digested with *Nsi*I, *Bgl*II, and *Xba*I. *Bgl*II lowered the background of the plasmids that were digested by only *Nsi*I or *Xba*I. pWLG18 was digested with *Nsi*I and *Xba*I, and the *Nsi*I-*Xba*I fragment containing *lacZ* was gel purified. After ligation and transformation into *E. coli* XL1-Blue MRF′, pWLG30 + *lacZ* was found by screening for blue colonies on LB + ampicillin + X-gal plates. The sequencing primers designed for confirming cloned DNA in pWLG30 or pWLG30 + *lacZ* were 5′-ACTCTCCAGAATACATAAAA-3′ (pWLG30 seq1) and pMEB.2seq2.

Assays of AHAS activity: The wild-type strain JJ1 or a strain transformed with pWLG13 + 5′-*ilvB* was grown in 100 ml of McNA in modified Wheaton bottles at 100 kPa of H_2:CO_2 to A_{600} = 0.60–0.75. The cells were harvested by centrifugation under anaerobic conditions (Shieh *et al.* 1988). The pellets were resuspended in 100 μl of dilution solution per 100 ml of medium. The dilution solution, which was sparged with N_2 for 1 hr, contained 1 mm cysteine hydrochloride, 1 mm dl-dithiothreitol, 1 mg bovine pancreatic DNase I (Boehringer Mannheim), 7.5 ml NaCl solution (293 g liter^{-1}), 50 ml distilled water, and 50 ml general salts solution (Whitman *et al.* 1986). The cells were placed in an aluminum seal vial on ice and flushed with hydrogen gas for 2 min before freezing at −20° for 2 hr. Upon thawing, the cells had lysed, and the cell extract was dialyzed in 12,000–14,000 molecular weight cut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Nru I
EcoR V
Xba I
Xba I
Pvu II
Plac
ori
Pmcr
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Sac I
Pvu II
Nde I
Bgl II
EcoR I
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Mipuid
9.37 kb
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Xma I
EcoR I
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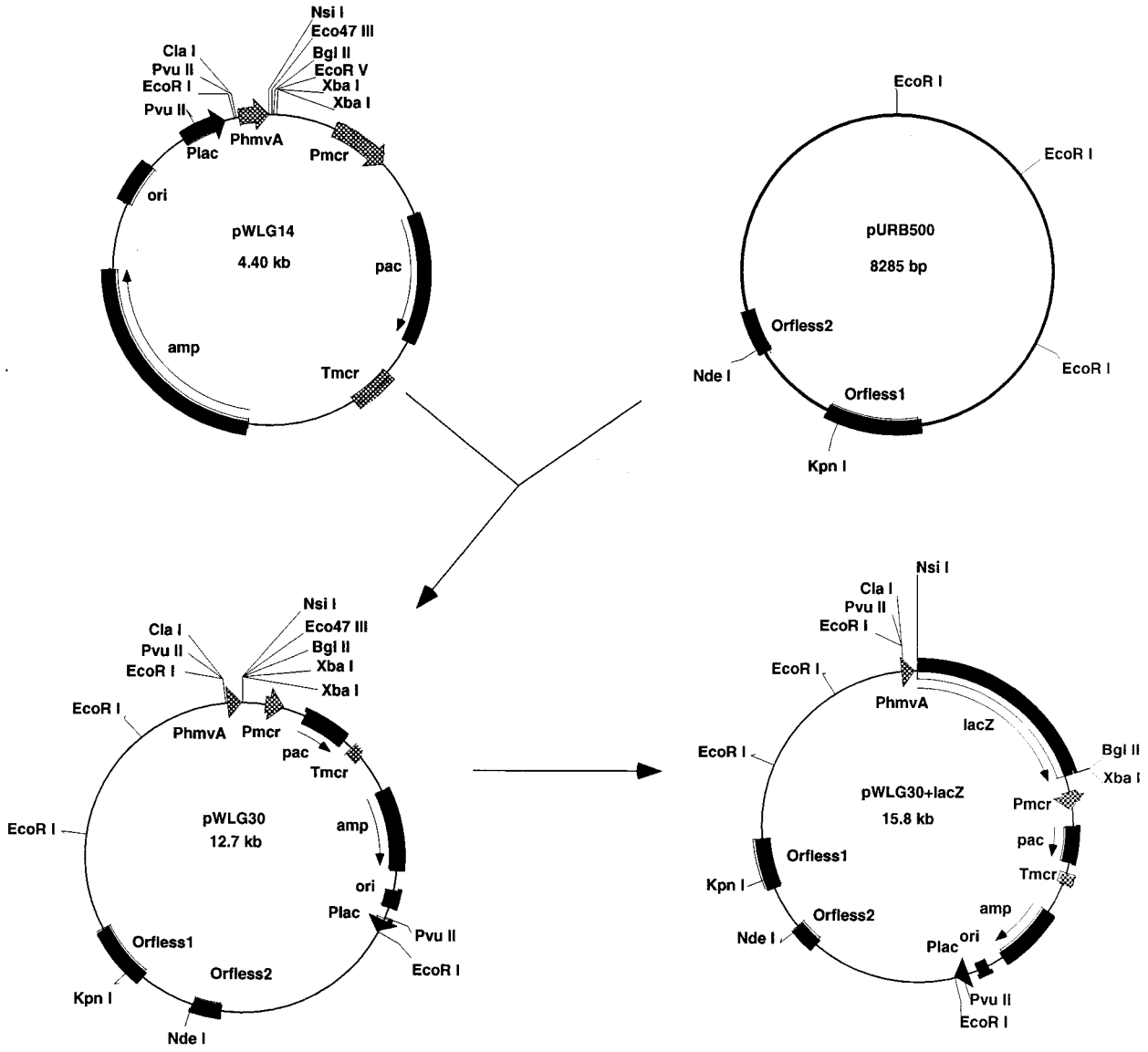

Figure 2.—Construction of methanococcal expression shuttle vectors pWLG30 and pWLG30 + *lacZ*. pWLG30 was constructed by ligation of a partial *Eco*RI digestion of the cryptic methanococcal plasmid pURB500 into the *Eco*RI site of pWLG14. *lacZ* from pWLG18 (Figure 1) was then added between the unique *Nsi*I and *Bgl*II sites of pWLG30. The low level of *lacZ* expression of pWLG30 + *lacZ* forms a blue/white screen for clones in *E. coli*. The unique *Cla*I site in pWLG14 and pWLG30 allows the P_{hmvA} to be replaced with other promoters in future studies.

off (MWCO) tubing overnight at 4°. The dialysis buffer was the basic purification buffer that had been sparged with N_2 and autoclaved (Xing and Whitman 1994). The AHAS assays were performed at 37° as described previously (Park *et al.* 1995). For protein quantification by the bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL), the dialyzed extract was first diluted 1:50 in 0.1 m sodium hydroxide, boiled for 10 min, and cooled on ice.

Assays of β-galactosidase activity: The β-galactosidase assay was performed with 2-nitrophenyl-β-d-galactopyranoside

Figure 1.—Construction of the integrative vectors pWLG13, pWLG14, and pWLG18. Only the unique or otherwise useful restriction sites are shown. pMEB.2 was digested with *Nde*I and *Sac*I to remove *lacZ′* before a MCS was added to form pWLG11. pWLG14 was constructed by the addition of P_{hmvA} between the *Eco*RI and *Bgl*II sites of pWLG11. *lacZ*, modified by PCR to contain *Nsi*I and *Bgl*II restriction sites, was ligated into pWLG14 to form pWLG18. pWLG12A was developed from pWLG11 by the addition of P_{hmvA} and *uidA* from Mipuid. The *uidA* was released from pWLG12A by a *Nde*I digestion to form pWLG13. The MCS was designed so that multiple restriction enzymes can be utilized in a single buffer. Using either *Nde*I or *Nsi*I plus a second restriction enzyme allows directed cloning into the vectors. Addition of a third endonuclease with a site between the first endonuclease sites can lower the background associated with the religation of a single digestion. P_{hmvA} can also be replaced using *Eco*RI (or *Cla*I in pWLG14) and *Nsi*I or *Nde*I.

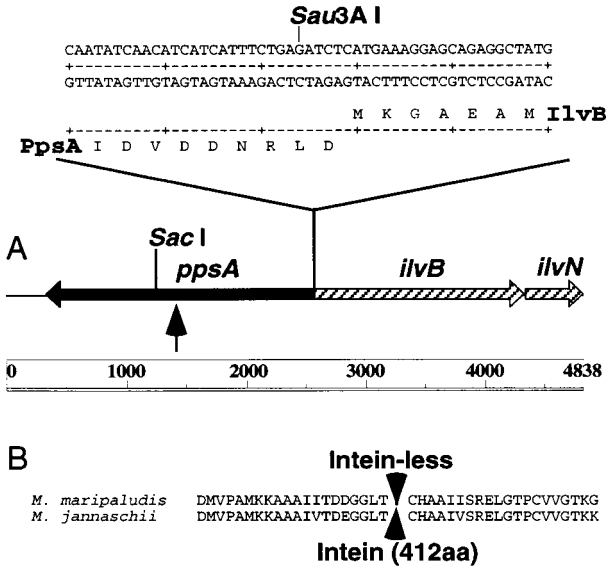

Figure 3.—(A) Sequenced region of pWLG1 containing the *M. maripaludis* genes encoding AHAS (*ilvBN*) and phosphoenolpyruvate synthase (*ppsA*). The junction between *ppsA* and *ilvB* indicating the position of the *Sau*3AI site likely to be involved in formation of a chimera is shown. The arrow corresponds to the position of the putative intein splice junction in the *M. jannaschii ppsA*. (B) Deduced amino acid sequence of PpsA from *M. maripaludis* and *M. jannaschii* near the intein splice junction. Arrow represents putative intein splice junction.

(ONPG) at a temperature of 37° (Miller 1992). *E. coli* XL1-Blue MRF′ containing pWLG30+*lacZ* was grown overnight in low salt LB broth medium/ampicillin without IPTG. These cells were inoculated into fresh LB broth/ampicillin with and without 1 mm IPTG and grown to OD_{600} = 0.64–0.70 before being assayed. *M. maripaludis* was grown on McC medium/puromycin unless stated otherwise. *M. maripaludis* assays used the same conditions as *E. coli*.

SDS-PAGE: This procedure was performed according to the method of Laemmli (1970) for a 10% polyacrylamide gel.

Nucleotide sequence accession numbers: The GenBank accession nos. for the *M. maripaludis* AHAS gene (*ilvBN*) and *ppsA* are AF118061 and AF118060, respectively. The accession nos. for pWLG11, pWLG13, pWLG14, and pWLG30 are AF134196, AF134197, AF134198, and AF134199, respectively.

RESULTS AND DISCUSSION

Cloning of the *ilvBN* genes of *M. maripaludis*: The two subunits of AHAS, which catalyze an early step in branched-chain amino acid biosynthesis, are encoded by the *ilvBN* genes in *M. aeolicus* (Bowen *et al.* 1997). Screening of the *M. maripaludis* genomic library identified one plaque that hybridized with the *M. aeolicus ilvB* probe. Upon conversion of the phagemid to the plasmid form, the plasmid pWLG1 was found to contain ~7 kbp of *M. maripaludis* genomic DNA. A total of 4838 bp was sequenced, and three ORFs with homology to known genes were identified (Figure 3A). Two ORFs encoded the large and small AHAS subunits (E.C. 4.1.3.18), *ilvB* and *ilvN*, respectively. The deduced amino acid sequence of the large subunit (IlvB), which contained 587 amino acids, had 68.1, 72.9, and 31.4% sequence identity to the *M. aeolicus*, *M. jannaschii* (MJ0227), and *M. jannaschii* (MJ0663) IlvB homologs, respectively. The low amino acid sequence similarity to MJ0663 was consistent with the assignment of this ORF to another function (Bowen *et al.* 1997). Fourteen nucleotides separated *ilvB* from *ilvN*. Upon comparison to the *M. aeolicus* gene, *ilvN* appeared to be truncated at the 3′ end to form a deletion equivalent to eight amino acids. This conclusion was supported by the presence of a *Sau*3A I site and the beginning of the pBK-CMV vector before a stop codon in the *ilvN* sequence. The deduced amino acid sequence of the small subunit (IlvN) from *M. maripaludis* was 71 and 73% identical to the *M. aeolicus* and *M. jannaschii* IlvN homologs, respectively. The *ilvBN* genes from *M. aeolicus*, which were separated by 172 bp, and *M. maripaludis* were linked (Bowen *et al.* 1997). In contrast, the *M. jannaschii* AHAS genes were unlinked (Bult *et al.* 1996).

Enzyme activity corresponding to a phosphoenolpyruvate synthase (E.C. 2.7.9.2; pyruvate, water dikinase) has been described in the cellular extracts of *M. maripaludis* (Shieh and Whitman 1987), and *ppsA* appeared immediately upstream of *ilvB*. When compared to the *M. jannaschii*, *Pyrococcus furiosus*, and *Staphylothermus marinus* homologs, the *M. maripaludis* PpsA was between 29 and 38 amino acids shorter at the N terminus. In addition, an initiation codon was not observed at the 5′ end of the cloned ORF, but a *Sau*3AI site was present, suggesting that the cloned *ppsA* had been truncated and that the cloned DNA in pWLG1 was chimeric (Figure 3A). Alternatively, it is also possible that the *ppsA* cloned was a pseudogene naturally present in the genome. In contrast, *ilvB* did not appear to be truncated because the 5′ end of the gene encoded an amino acid sequence nearly identical to the N-terminal sequence of the *M. aeolicus* AHAS (Xing and Whitman 1994). At the amino acid level the *M. maripaludis* PpsA was 72% identical to the *M. jannaschii* PpsA (MJ0542). The *M. jannaschii ppsA* contained an internal insertion that translated into a putative intein of 412 amino acids (Bult *et al.* 1996). At the intein/extein junction, a mechanistically important cysteinyl residue was found in the first position of the second extein (Bult *et al.* 1996; Chong *et al.* 1996; Figure 3B). The deduced amino acid sequence of the *M. maripaludis* PpsA was nearly identical to the *M. jannaschii* homolog near the intein/extein junction, and the mechanistically important cystienyl residue was conserved. The putative intein, however, was absent.

Overexpression of *ilvBN* in *M. maripaludis*: AHAS is normally expressed at the low levels typical of biosynthetic genes within the methanococci (Xing and Whitman 1987). Because this enzyme is both unstable and O_2 labile, it would be much easier to characterize biochemically if it could be overexpressed. To overexpress this gene, pWLG13+5′-*ilvB* was constructed. In this plas-

TABLE 2

Overexpression of the acetohydroxyacid synthase in *M. maripaludis* transformed with pWLG13 + 5′*ilvB*

Vector	Specific activity[a]
None	0.011
pWLG13 + 5′ *ilvB*	0.150

Cells were grown to an absorbance of 0.7–0.9 in McNA.
[a] Micromoles of acetolactate formed per minute per milligram of protein. Average of triplicate assays.

TABLE 3

Expression of β-galactosidase in *M. maripaludis* by the shuttle vector

Vector	Specific activity[a]
pWLG30	<0.02[b]
pWLG30 + *lacZ*	4.5

Cells were grown to an absorbance of 0.45–0.48 in McC medium/puromycin.
[a] Micromoles per minute per milligram of protein. Average of duplicates.
[b] No activity was detected.

mid, the start codon of *ilvB* overlapped with the *Nde*I site 4 bp downstream of the ribosome-binding site and promoter (P_{hmvA}) for the *M. voltae* histone (Figure 1). This plasmid also contained 641 bp of the 5′ end of *ilvB* to allow for efficient homologous recombination into the genomic copy of *ilvB*. After transformation into *M. maripaludis*, integration of the pWLG13+5′-*ilvB* into the genome would place a truncated *ilvB* gene under control of the native promoter and place the intact *ilvBN* operon under the control of P_{hmvA}. To test this hypothesis, the specific activity of AHAS in extracts of a pWLG13+5′-*ilvB* transformant was determined (Table 2). Compared to extracts of wild-type cells, AHAS specific activity was 13-fold higher in the transformant. The anticipated specific activity was calculated from the expression of the *Methanothermus fervidus* histone. In this organism, the number of histone molecules was 1–2 × 10^4 molecules per genome, and the genome possessed two genes (Stroup and Reeve 1992). The level of expression per gene would then be expected to be ~0.5–1 × 10^4 molecules. On the basis of the specific activity and molecular weight of the *M. aeolicus* AHAS (Xing and Whitman 1994), the same level of expression of the *M. maripaludis ilvBN* operon would yield a specific activity of 0.08–0.17 units (mg of protein)$^{-1}$ or very close to the observed value. This result also demonstrated that the *M. voltae* promoter was active in *M. maripaludis*.

To facilitate cloning into the integration vectors, pWLG18 was constructed with *lacZ* downstream of the P_{hmvA} to provide an opportunity for blue/white screening of clones in *E. coli* (Figure 1). Because *lacZ* contains an *Nde*I site, pWLG18 was constructed with an *Nsi*I site immediately downstream of the P_{hmvA}, overlapping the start codon. The availability of a vector with an *Nsi*I site also provides an alternative method for cloning genes that possess *Nde*I sites near their 5′ end and that would not be suitable for cloning in pWLG13.

Construction of the expression shuttle vector: To express heterologous genes in *M. maripaludis*, an expression shuttle vector was constructed from pWLG14 and pURB500 (Figure 2). pURB500 is a cryptic plasmid from *M. maripaludis* C5 and contains a methanococcal origin of replication, an *Nde*I site, and three *Eco*RI sites (Tumbula *et al.* 1997). The *Nde*I site precluded use of this same site near the P_{hmvA}. Therefore, pWLG14 was chosen because it contained an *Nsi*I site downstream of P_{hmvA}. The expression vector was then constructed by ligation of a partial *Eco*RI digestion of pURB500 into pWLG14 and transformation of *M. maripaludis* to select for the methanococcal origin of replication and puromycin resistance. Plasmids isolated from two independent transformants contained the same *Sac*II and *Eco*O109I endorestriction maps, indicating that pWLG14 was inserted in the same *Eco*RI site of pURB500, as utilized in pDLT44 (Figure 2, Tumbula *et al.* 1997). The expression shuttle vector pWLG30 was then further modified by adding *lacZ* to allow for blue/white screening in *E. coli* (Figure 2).

E. coli XL1-Blue MRF′ containing pWLG30 + *lacZ* produced blue colonies in the presence of X-gal. The production of β-galactosidase was independent of the addition of IPTG, indicating that expression was not from the *lac* promoter. Enzymatic assays of *E. coli* in LB broth plus ampicillin detected β-galactosidase activity slightly above background levels. Weak expression with P_{hmvA} in *E. coli* was also observed by Beneke *et al.* (1995) using Mipuid. Like pDLT44, pWLG30 transformed *M. maripaludis* with a high efficiency even though the plasmid yields were low from *E. coli*. pWLG30, isolated from *M. maripaludis*, was checked for rearrangements after transformation back into *E. coli*. No rearrangements were observed.

Expression of *lacZ* in *M. maripaludis* and *E. coli*: The expression shuttle vector pWLG30 was tested for *in vivo* expression of the β-galactosidase gene (*lacZ*) from *E. coli*. *M. maripaludis* does not contain β-galactosidase, and activity was not detectable in wild-type cells (Cohen-Kupiec *et al.* 1997). However, β-galactosidase was readily detectable in cell-free extracts of a transformant bearing pWLG30 + *lacZ* (Table 3). A protein band corresponding to the expected size of β-galactosidase was found by SDS-PAGE in the transformants of pWLG30 + *lacZ*, but not in cells transformed with pWLG30 alone (Figure 4).

The specific activity of β-galactosidase varied with the growth phase of the culture. During growth on H_2:CO_2, exponential growth was usually observed only at low cell

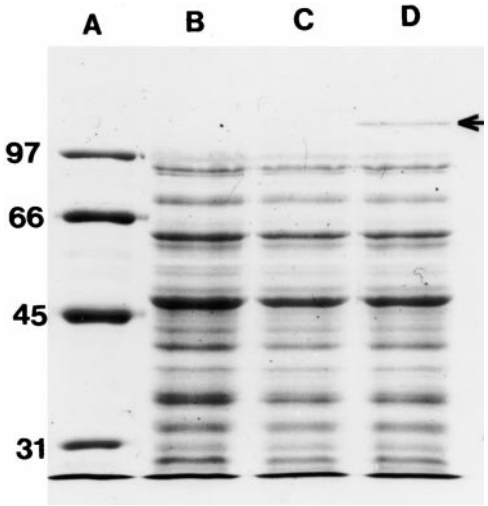

Figure 4.—Overexpression of β-galactosidase in *M. maripaludis* by the expression shuttle vector pWLG30. SDS-PAGE of total protein from *M. maripaludis* stained with Coomassie blue. Bio-Rad low-molecular-weight standards (lane A): rabbit muscle phosphorylase b (97.4 kD), bovine serum albumin (66.2 kD), hen egg white ovalbumin (45.0 kD), and bovine carbonic anhydrase (31.0 kD). The transformants were grown in McC medium/puromycin to an absorbance of 0.5, and the wild type was grown to an absorbance of 0.8 in McC medium. Lane B, wild-type cells; lane C, cells transformed with pWLG30; lane D, cells transformed with pWLG30+*lacZ*; arrow is a protein with the expected M_r of β-galactosidase (116.3 kD).

densities, *i.e.*, $A_{600} < 0.4$ (Jones *et al.* 1983b). At higher cell densities, the rate of H_2 transfer from the gas to liquid phases probably limited growth, and arithmetic growth was observed (Figure 5A). In cultures of the transformant, the specific activity of β-galactosidase increased threefold from exponential to stationary phase. During the stationary phase, where absorbance (600 nm) = 1, the β-galactosidase specific activity was 7.5 μmol min^{-1} (mg of protein)$^{-1}$. Purified β-galactosidase has a specific activity of 600 μmol min^{-1} (mg of protein)$^{-1}$ (Boehringer Mannheim). Thus, the β-galactosidase appeared to represent ~1% of the cellular protein of the transformant. Whether or not the increase in specific activity resulted from changes in the level of expression from P_{hmvA} or in the copy number of the plasmid was not determined.

The stability of pWLG30 in methanococci is important for the production of large amounts of recombinant protein. In one experiment, pWLG30 + *lacZ* was transferred every 3 wk in McC+puromycin medium for 3 mon. Upon electroporation into *E. coli*, all the >500 colonies examined on LB-amp + X-gal-containing medium were blue, indicating that the *lacZ* had been maintained. Restriction endonuclease mapping of the plasmid from one of the clones failed to detect any differences with the original plasmid. To further address this issue, the levels of β-galactosidase were tested after multiple transfers in media without puromycin (Figure

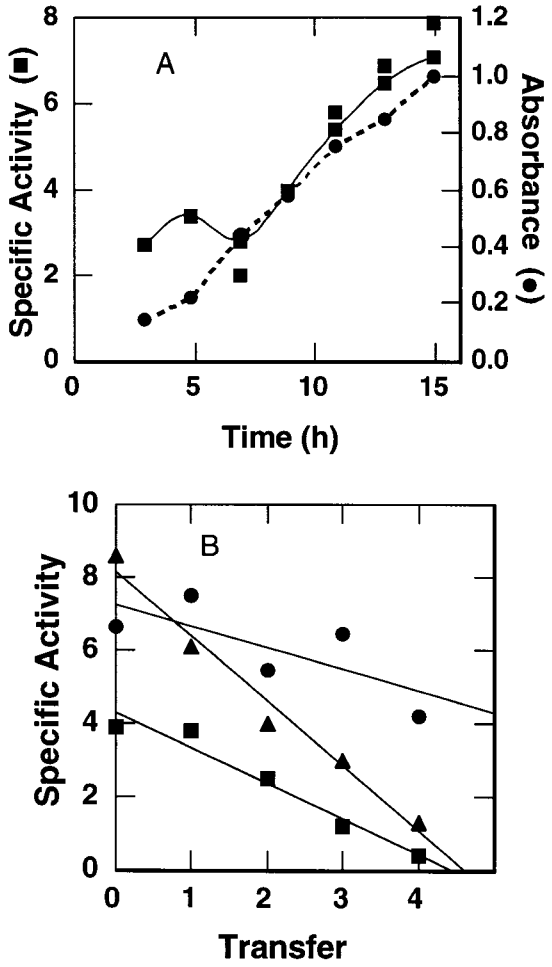

Figure 5.—Expression of β-galactosidase in a transformant of pWLG30 + *lacZ*. Specific activity was in micromoles of X-gal transformed min^{-1} (mg of protein)$^{-1}$. (A) Effect of growth phase on the specific activity. Cells, 4% v/v, were inoculated into McC/puromycin medium. (B) Stability of pWLG30 + *lacZ* in *M. maripaludis* upon serial transfers of the culture. *M. maripaludis* was grown to an absorbance (600 nm) of 0.62–0.85 in McN (■), 0.40–0.58 in McC (▲), and 0.44–0.66 in McC/puromycin (●). The inoculum was 4%. Every 24 hr, the culture was transferred to fresh medium, and the specific activity was determined after 14 hr of growth. The average number of generations in the three media after transfers 1, 2, 3, and 4 were 5, 10, 14.5, and 23, respectively. Data points were the average of duplicate assays. For McN, McC, and McC/puromycin media, correlation coefficients of the specific activity with the number of transfers were 0.99, 0.98, and 0.74, respectively. Critical values for significance at $P = 0.05$ and 0.01 were 0.88 and 0.96, respectively.

5B). In both mineral (McN) and rich (McC) media, the levels of β-galactosidase activity rapidly dropped after four serial transfers in media without puromycin. Although the initial specific activity in mineral medium was lower than in complex medium, the relative rate at which activity was lost was ~20–25% per transfer in both media. If the loss in β-galactosidase activity was an indication of loss of the plasmid, the availability of the amino acids and other components of the rich medium

appeared to have little affect on this process. In contrast, in the presence of puromycin, the level of β-galactosidase activity was maintained for at least four transfers, and the apparent decline was not statistically significant (Figure 5B).

Summary: To our knowledge, this report is the first description of an expression shuttle vector for the methanogenic archaea. Because these strict anaerobes contain many oxygen-sensitive enzymes and unusual coenzymes, they are good candidates for an expression system for enzymes that are not expressed in an active form in *E. coli*. Among the methanogens, the methanococci may be especially useful in this regard. Their rapid growth facilitates genetic manipulations. They can be cultured on a large scale on formate, which substantially reduces the hazards and expense of cultivation with H_2:CO_2. Thus, even though the levels of expression are only ~1% of the total cellular protein, it is possible to obtain large amounts of protein.

In addition to expressing homologous enzymes for biochemical studies, the integrative expression vectors may be useful for manipulating the physiology of *M. maripaludis*. For instance, a spontaneous mutation in *Methanosarcina barkeri* overexpressed the genes encoding pyruvate oxidoreductase and allowed the mutant to grow on pyruvate as an electron donor (Bock and Schönheit 1995). With the integrative expression vector, it would be possible to recreate such a mutant in *M. maripaludis* to determine if it also allows heterotrophic growth on pyruvate.

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