JOURNAL OF BACTERIOLOGY, May 2000, p. 2611–2618 Vol. 182, No. 9
0021-9193/00/$04.00+0

Generation of Dominant Selectable Markers for Resistance to Pseudomonic Acid by Cloning and Mutagenesis of the *ileS* Gene from the Archaeon *Methanosarcina barkeri* Fusaro

PAOLO BOCCAZZI, JUN KAI ZHANG, AND WILLIAM W. METCALF*

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

Received 13 September 1999/Accepted 26 January 2000

Currently, only one selectable marker is available for genetic studies in the archaeal genus *Methanosarcina*. Here we report the generation of selectable markers that encode resistance to pseudomonic acid (PA^r) in *Methanosarcina* species by mutagenesis of the isoleucyl-tRNA synthetase gene (*ileS*) from *Methanosarcina barkeri* Fusaro. The *M. barkeri ileS* gene was obtained by screening of a genomic library for hybridization to a PCR fragment. The complete 3,787-bp DNA sequence surrounding and including the *ileS* gene was determined. As expected, *M. barkeri* IleS is phylogenetically related to other archaeal IleS proteins. The *ileS* gene was cloned into a *Methanosarcina-Escherichia coli* shuttle vector and mutagenized with hydroxylamine. Nine independent PA^r clones were isolated after transformation of *Methanosarcina acetivorans* C2A with the mutagenized plasmids. Seven of these clones carry multiple changes from the wild-type sequence. Most mutations that confer PA^r were shown to alter amino acid residues near the KMSKS consensus sequence of class I aminoacyl-tRNA synthetases. One particular mutation (G594E) was present in all but one of the PA^r clones. The MIC of pseudomonic acid for *M. acetivorans* transformed with a plasmid carrying this single mutation is 70 μg/ml of medium (for the wild type, the MIC is 12 μg/ml). The highest MICs (560 μg/ml) were observed with two triple mutants, A440V/A482T/G594E and A440V/G593D/G594E. Plasmid shuttle vectors and insertion cassettes that encode PA^r based on the mutant *ileS* alleles are described. Finally, the implications of the specific mutations we isolated with respect to binding of pseudomonic acid by IleS are discussed.

In recent years, there has been significant progress in developing methods of genetic analysis applicable to methanoarchaea. Among the most important of these are the development of simple and reliable methods for growth and isolation of clonal populations (23, 42), the development of a functional selectable marker for puromycin resistance (16), the development of plasmid shuttle vectors (29, 45), and the development of highly efficient transformation protocols for *Methanosarcina* and *Methanococcus* species (29, 45).

Although the ability to select puromycin-resistant transformants has revolutionized genetic analysis among the methanoarchaea, few other selectable markers have been subsequently developed. This dearth of usable selectable markers is unfortunate because many genetic studies require multiple selectable markers, for example, the introduction of plasmids into strains that already carry a chromosomal insertion of one selectable marker. Selection for histidine prototrophy or neomycin resistance is possible in certain *Methanococcus* species (2, 35); however, neither of these have been shown to be useful in the *Methanosarcina* species under study in our laboratory. We have demonstrated that neither neomycin nor the related antibiotic G-418 is effective against *Methanosarcina* spp., even at a concentration of 1 mg/ml (P. Boccazzi and W. W. Metcalf, unpublished data). Indeed, few antibiotics that are effective against bacteria are effective against archaea (6). To complicate matters further, selectable markers that might confer resistance to the few effective antibiotics are lacking. This problem is exemplified by the antibiotic chloramphenicol, which is highly effective against many methanoarchaea. In bacteria, resistance to chloramphenicol can be achieved by acetylation of the antibiotic. Unfortunately, acetylchloramphenicol is an equally effective antibiotic among the methanoarchaea. Thus, the *cat* gene, encoding chloramphenicol acetyltransferase, is not expected to confer resistance on methanoarchaea (4). We have verified this in vivo by cloning the *cat* gene under the transcriptional control of a strong *Methanosarcina* promoter into a plasmid shuttle vector. *Methanosarcina* transformants carrying this plasmid were shown to express active Cat protein but were not resistant to either chloramphenicol or fusaric acid (M. A. Pritchett and W. W. Metcalf, unpublished data).

An attractive candidate for development of a usable selectable marker is the antibiotic pseudomonic acid A. Pseudomonic acid is an antibiotic produced by certain strains of *Pseudomonas fluorescens* (15). The antibiotic is an analog of the amino acid isoleucine that inhibits protein synthesis by blocking isoleucine charging to its cognate tRNA by isoleucyl-tRNA synthetase (19, 20, 47). Pseudomonic acid is an effective antibiotic against many methanoarchaea (21, 36). Importantly, mutants of *Methanobacterium thermoautotrophicum* that are resistant to pseudomonic acid (PA^r) have been isolated (21). The mutations found in these resistant strains were shown to map to the *ileS* gene of this organism, which encodes the target of the antibiotic, isoleucyl-tRNA synthetase. These mutations could be transferred by phage transduction, indicating that, at least in this species, mutant *ileS* alleles can be used as selectable markers (26). However, it was unclear from these data whether the mutant *ileS* alleles would confer resistance on strains that also carry a wild-type allele of *ileS*, such as might occur if the genes were used as selectable markers on a plasmid construct. In *Staphylococcus aureus*, PA^r is conferred by a plasmid-encoded IleS protein (13). The fact that this plasmid can be transferred to sensitive strains (that presumably carry an antibiotic-sensitive *ileS* gene), with concomitant transfer of

* Corresponding author. Mailing address: Department of Microbiology, University of Illinois, B103 Chemical and Life Science Laboratory, 601 S. Goodwin, Urbana, IL 61801. Phone: (217) 244-1943. Fax: (217) 244-6697. E-mail: metcalf@uiuc.edu.

PA^r, suggests that resistant *ileS* alleles are dominant over sensitive alleles (33). Therefore, we reasoned that mutagenesis of a native *ileS* gene from *Methanosarcina* spp. might be used to develop an alternative selective marker for members of this genus. In this paper, we report the cloning and sequencing of the *ileS* gene from *Methanosarcina barkeri* Fusaro and its modification to allow its use as a selectable marker for PA^r in *Methanosarcina acetivorans* C2A.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Standard conditions were used for growth of *Escherichia coli* strains (46). Strains DH5α and DH5α/λ*pir* (32) were from S. Maloy (University of Illinois). Strain DH5α was used as the host for plasmid constructions using pTZ18R (Pharmacia, Piscataway, N.J.) or pBluescript KS(+) (Stratagene, La Jolla, Calif.) as a cloning vector. Strain DH5α/λ*pir* or WM95 (28) was used as the host for construction of *pir*-dependent replicons. *M. acetivorans* C2A (= DSM 2834) and *M. barkeri* Fusaro (= DSM 804) were from laboratory stocks. *Methanosarcina* strains were grown in single-cell morphology (42) at 35°C in HS-methanol-acetate (HS-MA) broth medium under strictly anaerobic conditions (30). Growth of *Methanosarcina* strains on solid medium was done essentially as described in reference 42, with the exception that *M. acetivorans* could be plated without the use of top agar by spreading or streaking directly on the surface of agar-solidified medium (*M. barkeri* Fusaro was plated in top agar as described in reference 42). All plating manipulations were carried out under strictly anaerobic conditions in an anaerobic glove box. Plates with inoculated solid medium were incubated in an intrachamber anaerobic incubator as described in reference 31. Puromycin was used at 1 or 2 μg/ml for *Methanosarcina* species. Pseudomonic acid A, a gift from SmithKline Beecham Pharmaceuticals (Philadelphia, Pa.), was routinely used at 35 μg/ml, except as noted otherwise. Puromycin and pseudomonic acid were added to medium from sterile, anaerobic stock solutions. Stock solutions were sterilized through NALGENE 0.2-μm-pore-size filters (Nalge Company, Rochester, N.Y.) into sterile, sealed serum bottles and then made anaerobic by applying vacuum for three cycles of 15 min each. Inquiries regarding the availability of pseudomonic acid should be forwarded to P. G. Treagust, manager, Reference Materials Group, SmithKline Beecham Pharmaceuticals.

DNA methods. Standard methods were used throughout for isolation and manipulation of DNA (3). Plasmid DNA was isolated from *Methanosarcina* species as previously described (29). Alternatively, if the plasmids present in *Methanosarcina* strains were to be used solely for transfer to *E. coli*, the cells were lysed by resuspension in sterile H_2O and the plasmid DNA was concentrated by precipitation and then used directly for transformation. Genomic DNA of high molecular weight was isolated from *Methanosarcina* species as follows. Cells from a stationary-phase culture grown in 200 ml of HS-MA medium were collected by centrifugation, resuspended in 10 ml of 50 mM Tris-HCl (pH 8.0) with 0.85 M sucrose, and lysed by addition of sodium dodecyl sulfate to a final concentration of 0.5%. The lysate was treated with proteinase K (150 μg/ml) at 37°C for 3 h, extracted three times with buffered phenol-$CHCl_3$-isoamyl alcohol (25:24:1), and then extracted once with $CHCl_3$-isoamyl alcohol (24:1). Finally, the DNA was precipitated from the treated, extracted lysate with NaCl and isopropanol; excess salt was removed by rinsing of the precipitate sequentially with 70% ethanol (three times) and 100% ethanol. The precipitated DNA was then dried and resuspended in 10 mM Tris-HCl (pH 8.0) with 1 mM EDTA. DNA hybridizations were performed as previously described (30). Colony hybridizations were performed as described in reference 3. Probes used for hybridization experiments were labeled with [α-^{32}P]dATP using the Prime-a-Gene kit (Promega, Madison, Wis.) according to specifications. DNA sequences were determined from double-stranded templates by automated dye terminator sequencing. DNA sequencing was performed at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois.

Transformations. *E. coli* was transformed by electroporation using an *E. coli* Gene Pulser (Bio-Rad, Richmond, Calif.) as recommended. *Methanosarcina* species were transformed by liposome-mediated transformation essentially as described in reference 29, except that the transformations were carried out in an anaerobic chamber with an atmosphere of N_2-CO_2-H_2 (75:20:5) and that anaerobic HEPES buffer was replaced by sterile anaerobic sucrose (0.85 M) buffered with $NaHCO_3$ (80 mM). The pH of the buffered sucrose solution was 7.4 under a 20% CO_2 atmosphere.

Construction of the *Methanosarcina-E. coli* shuttle cosmids pWM348 and pWM349. The shuttle cosmids pWM348 and pWM349 were constructed by cloning the dual *cos* sites and polylinker from Supercos (Stratagene) into the *Methanosarcina-E. coli* shuttle vector pWM307 (29). The *cos* sites and polylinker were PCR amplified from Supercos using *Taq* DNA polymerase and the primers 5′-GCC**GGCGCGCC**TATAAAAATAGGCGTATCACGAGG-3′ and 5′-GCC**GGCGCGCC**TTGAAGGCTCTCAAGGGCATCGGTCG-3′ (added *Asc*I sites are in boldface). The PCR product was digested with *Asc*I and cloned into the unique *Asc*I site of pWM307 to generate pWM348 and pWM349. The two plasmids differ only with respect to the orientation of the insert.

Cloning of an internal *ileS* gene fragment from *M. barkeri* Fusaro and *M. acetivorans* C2A. Internal *ileS* gene fragments were obtained from *M. barkeri* Fusaro and *M. acetivorans* C2A by PCR amplification from genomic DNA essentially as described in reference 7. The primers used for amplification were modified slightly to more closely match the archaeal consensus sequences and were 5′-GGNTGGGAYACNCAYGGNYTNCCNATHGA-3′ and 5′-GGNG TYTTRCANCKCCARCA-3′. Amplifications were performed using *Taq* DNA polymerase. The ca. 930-bp PCR products were made blunt by treatment with T4 DNA polymerase and deoxynucleoside triphosphates, phosphorylated by treatment with T4 polynucleotide kinase, and cloned into the *Sma*I site of pTZ18R to generate pWM351 and pWM352, which carry internal *ileS* fragments from *M. barkeri* Fusaro and *M. acetivorans* C2A, respectively. DNA sequence analysis of pWM351 and pWM352 strongly suggests that the two plasmids each carry fragments of the *ileS* gene of the respective *Methanosarcina* species.

Cloning and DNA sequence analysis of the complete *ileS* gene of *M. barkeri* Fusaro. A cosmid-based genomic library of *M. barkeri* Fusaro was constructed by ligation of *Sau*3AI-digested genomic DNA with *Nhe*I- and *Bam*HI-digested pWM348. After in vitro packaging, the library was transfected into DH5α/λ*pir* and ca. 4,000 independent Amp^r clones were saved for subsequent screening. A strain carrying the cosmid pJK63, which carries the intact *ileS* gene, was isolated from this library as a clone that hybridized to pWM351. Plasmid pJK64 was constructed by ligation of a 3,560-bp *Not*I-to-*Eco*RV fragment from pJK63 into *Not*I- and *Eco*RV-digested pBluescript KS(+). Plasmid pJK44 was constructed by ligation of a 3,812-bp *Not*I-to-*Sph*I DNA fragment containing the *M. barkeri* Fusaro *ileS* gene from pJK63 into the same sites in the *Methanosarcina-E. coli* shuttle vector pWM321 (29). The majority of the complete DNA sequence of the *M. barkeri* Fusaro *ileS* gene was determined from pJK63 and pJK64 templates; the remainder was determined from pJK44. Standard primers were used to generate junction sequences from these plasmids. Internal sequences were obtained with primers designed based on the junction and subsequent sequences.

Phylogenetic analysis of IleS sequences was performed as follows. Sequences were automatically aligned using the CLUSTAL W program (version 1.74) (43). Segments that were deemed confidently aligned were manually masked and then extracted using the AE2 alignment editor (T. Macke, University of Illinois at Urbana-Champaign). PAUP* (version 4 beta 2, D. Swofford, published by Sinauer Associates, Inc.) was used to generate the 500 most parsimonious trees from the masked amino acid alignment. These trees were evaluated using maximum-likelihood criteria with the ProtML program (MOLPHY software package [reference 1]). A consensus of the trees, standardized and exponentially weighted using the protein maximum-likelihood scores and the Kishino-Hasegawa test for significance, was generated using the TreeCons program (version 1.0; L. S. Jermiin and O. Anpilogova) and the CONSENSE program (J. Felsenstein; PHYLIP, phylogeny inference package, version 3.5c; Department of Genetics, University of Washington, Seattle, 1993). The resultant consensus tree is topologically equivalent to the best tree evaluated by maximum-likelihood analysis (22). Branch lengths were calculated by supplying the consensus tree to the ProtML program as a user tree.

Mutagenesis of *ileS*. Mutant forms of *ileS* that confer PA^r were generated by in vitro mutagenesis of pJK44 with hydroxylamine ($NH_2OH \cdot HCl$) using a protocol adapted from reference 25. The reaction mixture contained 0.8 M $NH_2OH \cdot HCl$, 100 mM $KHPO_4$, 1 mM EDTA, and 10 mM $MgSO_4$. Mutagenesis reactions were carried out in a 100-μl total volume containing ca. 2.0 μg of plasmid DNA. Independent reactions were incubated for 0, 6, 12, 18, 24, 36, or 42 h at 37°C. Subsequently, each sample was dialyzed for 6 h at 4°C by incubation of the reaction mixture on the surface of a 0.025-μm-pore-size filter (type VS; Millipore, Bedford, Mass.) floating on 20 ml of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer. Each sample was then precipitated and used in its entirety to transform *E. coli* DH5α/λ*pir* to Amp^r. All of the colonies from each transformation were pooled independently and used for large-scale plasmid isolations to obtain the quantities of DNA needed for *Methanosarcina* transformation. The mutagenized pJK44 pools were then used to transform *M. acetivorans* with selection for PA^r. A total of 12 mutagenized pools were screened by this method. From each of these pools, the first three colonies that appeared were purified by single-colony isolation on HS-MA agar with pseudomonic acid (35 μg/ml) and retained for subsequent analysis.

Subcloning and DNA sequence analysis. The 3,812-bp *Sph*I-*Not*I fragments containing the *ileS* gene from nine independent PA^r-encoding plasmids were subcloned into the same sites in pWM321 (29), generating pPB3, pPB4, pPB5, pPB6, pPB7, pPB22, pPB23, pPB24, and pPB29. This ensured that the PA^r phenotype associated with each mutant was the result of changes within the *ileS* region and not due to mutations within the vector backbone. The complete *ileS* DNA sequence present in each of these plasmids was then determined as described before.

Several additional plasmids were constructed that carried various combinations of the multiple mutations found in certain *ileS* mutants. Plasmid pPB8 was obtained by replacing the *Not*I-*Alw*NI DNA fragment of pPB4 with the same DNA fragment from pPB5. Plasmids pPB12 and pPB13 were obtained by replacing the *Eco*47III DNA fragment of pPB7 and pJK44, respectively, with the *Eco*47III DNA fragment of pPB5. Finally, the integration vector pPB18 was constructed by ligating the 3,812-bp *Sph*I-*Not*I DNA fragment of pPB12 into the same sites in pJK41 (27).

Phenotypic characterization. The MIC of pseudomonic acid and the generation times of *M. acetivorans* C2A strains carrying each mutant plasmid were determined by measuring the extent of growth in various media in terms of the optical density at 660 nm (OD_{660}). For the MIC experiment, duplicate sets of HS-MA medium containing pseudomonic acid at concentrations of 0, 35, 70, 140, 280, 560, and 1,120 μg/ml were inoculated with 100 μl (1%) of a late-log-phase (OD_{660}, ca. 0.5) culture grown in HS-MA broth plus puromycin. At this stage of growth, the inoculum was ca. 3×10^7 cells, which is well below the level expected to give rise to spontaneous PA^r mutants (spontaneous PA^r mutations occur in *M. acetivorans* with a frequency of $5.5 \pm 1.8 \times 10^{-9}$ [Boccazzi and Metcalf, unpublished]). A clone was considered PA^r if the measured OD was greater than 0.05 after 15 days of incubation at 37°C. Our data indicate that cultures which reached this OD ultimately grew to levels similar to that obtained by wild-type cells grown in the absence of antibiotic, although this often required substantially longer incubations. In all cases, the results obtained from replicate cultures were identical. Growth rates were determined in HS-MA medium containing pseudomonic acid (35 μg/ml) at 37°C. The inoculum was 100 μl (1%) of a late-log-phase culture grown in HS-MA plus puromycin (2 μg/ml). Generation times were calculated during the exponential growth phase (OD_{660} between 0.05 and 0.45). The reported data are averages of six trials.

Construction of PA^r-encoding *Methanosarcina-E. coli* shuttle vectors. A variety of *Methanosarcina-E. coli* shuttle vectors encoding PA^r were constructed in four steps as follows. First, pPB17 was constructed by self-ligation of a 1,482-bp *Eco*RI-digested PCR fragment amplified from pWM303 (29) using *Taq* DNA polymerase and the primers 5′-**GAATTCGCGGCCGCG**CTCTAGAGGCGCGC CAGGTGG-3′ and 5′-**GAATTCGCATGC**AATTCTGTCAGCCGTTAAGTG-3′ (added *Eco*RI, *Sph*I, and *Not*I restriction sites are in boldface). Plasmid pPB17 carries the origin of replication from plasmid R6K and the *bla* gene for replication and selection for ampicillin resistance in *E. coli*, respectively. Second, to allow replication of the constructs in *Methanosarcina*, the pC2A plasmid replicon was isolated as a 5,467-bp *Spe*I fragment from pWM241 (29) and cloned into the compatible *Xba*I site of pPB17 to generate pPB20. Third, to allow selection for PA^r in *Methanosarcina*, the 3,812-bp *Sph*I-*Not*I DNA fragment from pPB12 was cloned into the same sites of pPB20 to generate pPB26. Finally, to allow blue-white screening of recombinant clones, PCR fragments carrying the *lacZ*α gene and polylinker region amplified from pBluescript KS(+), pBluescript SK(+), and pMTL22 (12), respectively, using the primers 5′-GCC**GGCGCGCC**TTAAC CATTCGCCATTCAGGCTGC-3′ and 5′-GCC**GGCGCGCC**AATACGCAAA CCGCCTCTCC-3′ (added *Asc*I restriction sites are in boldface) were cut with *Asc*I and cloned into *Asc*I-digested pPB26 to generate pPB32, pPB33, and pPB35, respectively. Plasmids pPB31 and pPB34 are similar to plasmids pPB32 and pPB33, respectively, but carry the PCR fragment in the opposite orientation.

Nucleotide sequence accession number. The GenBank accession number for the *M. barkeri* Fusaro *ileS* gene and the surrounding sequence is AF208389.

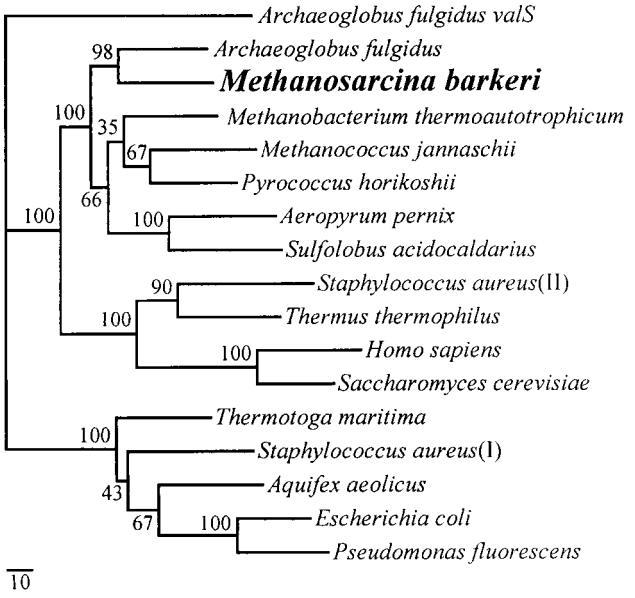

FIG. 1. Phylogeny of the *M. barkeri* IleS protein. The standardized, exponentially weighted consensus tree of the 500 most parsimonious trees, evaluated by maximum-likelihood analysis, is shown. A percentage at a node represents the relative-likelihood support for that branch, as calculated by the TreeCons program. The tree was rooted using the *valS* gene of *A. fulgidus*. The scale bar represents the estimated number of substitutions per 100 amino acid positions along each branch. The Swiss Protein database accession numbers of the sequences used are, from top to bottom, O28059 (*A. fulgidus valS*), O29622 (*A. fulgidus*), P26499 (*M. thermoautotrophicum*), Q58357 (*M. jannaschii*). P46215 (*S. acidocaldarius*), P41368 (*S. aureus* II, a plasmid-encoded copy that confers PA^r). P56690 (*T. thermophilus*), P41252 (*H. sapiens*), P09436 (*S. cerevisiae*), P46213 (*T. maritima*), P41972 (*S. aureus* I, genomic copy), P46207 (*A. aeolicus*), P00956 (*E. coli*), and P18330 (*P. fluorescens*). The *A. pernix* and *P. horikoshii* sequences were from the respective genome databases.

RESULTS

Cloning and sequence analysis of the *ileS* gene from *M. barkeri* Fusaro. A cosmid clone, pJK63, carrying the *ileS* gene of *M. barkeri* Fusaro was identified by hybridization to an internal *ileS* gene fragment obtained by PCR as described in Materials and Methods. A 3,787-bp DNA sequence including the putative *ileS* gene, as well as 437 bp upstream and 174 bp downstream, was subsequently determined. The putative *ileS* gene is predicted to encode a protein of 1,058 amino acids. This putative IleS protein is highly homologous to the known IleS proteins from other organisms, including archaea, eucarya, and bacteria (29.7 to 52.2% identical to the 25 closest homologs in the Swiss-Prot database). It also has several conserved IleS motifs, including ones that correspond to the HIGH and KMSKS "signature" sequences of class I aminoacyl-tRNA synthetases (10). The consensus sequence KMSKS is perfectly conserved in archaea, eucarya, and bacteria and is located at positions 596 to 600 of the *M. barkeri* Fusaro IleS. In the case of the HIGH motif, the observed sequence HLGT, located at positions 55 to 58, is the same as that found in the IleS sequences of the Archaea *Archaeoglobus fulgidus* (24), *M. thermoautotrophicum* (41), and *Methanococcus jannaschii* (8). Consistent with this observation, phylogenetic analysis of the *M. barkeri* IleS protein indicates that it is specifically related to the other archaeal IleS proteins (Fig. 1).

Two other sequence features are worthy of note. First, four copies of a 28-bp direct repeat, TTTTTTTAAAAGGGTTGC GGTCAAGCAG, are found about 300 bp upstream of the putative *ileS* gene. Interestingly, four copies of the same direct repeat are found at a similar location upstream of the *hdr* operon in this organism (17). The function of these repeat sequences is unclear; however, direct repeat sequences appear to be quite common in *Methanosarcina*. Second, a portion of an open reading frame was identified beginning 72 bp downstream of the *ileS* translation stop. This putative gene encodes a highly conserved archaeal protein of unknown function (the accession numbers of homologs from *M. jannaschii*, *M. thermoautotrophicum*, *Pyrococcus abyssi*, *A. fulgidus*, and *Pyrococcus horikoshii* are MJ1634, MTH1600, PAB1706, AF0878, and PH1244, respectively).

***ileS* gene mutagenesis.** We mutagenized the *M. barkeri* Fusaro *ileS* gene in the hope of creating alleles that are insensitive to the isoleucyl-tRNA synthetase inhibitor pseudomonic acid. To do this, the *ileS* gene was cloned into a *Methanosarcina-E. coli* shuttle vector and treated in vitro with hydroxylamine. Pools of mutagenized plasmids were then used to transform the related species *M. acetivorans* C2A with selection for PA^r (initially selected by using pseudomonic acid at 35 μg/ml). Preliminary experiments indicated that the MIC of pseudomonic acid for this species is approximately 12 μg/ml (W. W. Metcalf, unpublished data). Strains transformed with the *ileS* shuttle vector pJK44 (Fig. 2; Table 1) exhibit similar sensitivity to the antibiotic. Further, spontaneous resistance occurs at a relatively low frequency of $5.5 \times 10^{-9} \pm 1.8 \times 10^{-9}$. However, when *M. acetivorans* was transformed with the mutagenized plasmid pools, numerous PA^r colonies were obtained. Because we were interested in obtaining mutants that conferred the highest levels of antibiotic resistance, we routinely checked

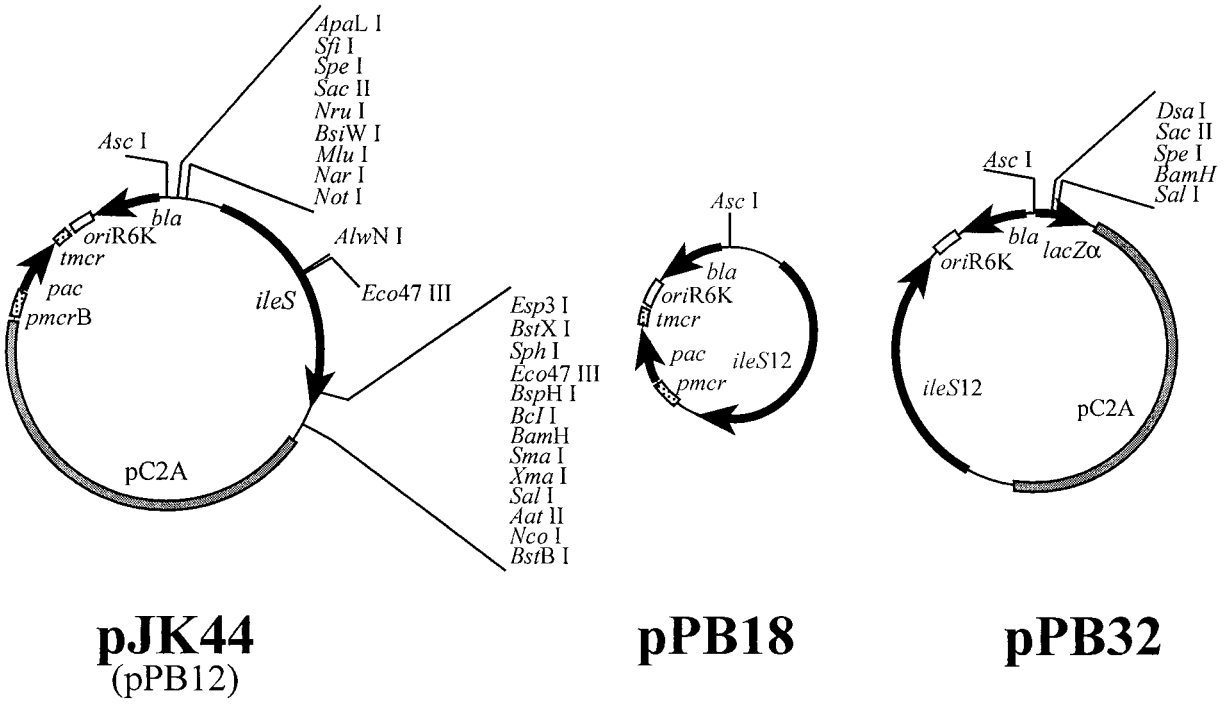

FIG. 2. Plasmids used in this study. Plasmid pJK44 carries the *M. barkeri* strain Fusaro *ileS* gene in *Methanosarcina-E. coli* shuttle vector pWM321 (29). The origin of replication from plasmid R6K (*ori*R6K) allows plasmid replication in *E. coli*, and the pC2A replicon allows replication in the genus *Methanosarcina*. The *pac* cassette confers puromycin resistance upon methanoarchaea, and the β-lactamase gene *bla* encodes resistance to ampicillin on *E. coli*. Plasmid pPB12 is identical to pJK44, except that it carries the *ileS12* allele in the place of wild-type *ileS*. Plasmid pPB18 differs from pPB12 by lacking the pC2A replicon and therefore is incapable of replication in *Methanosarcina*. Plasmid pPB32 is a *Methanosarcina-E. coli* shuttle vector that carries *ileS12*, *lacZ*α for blue-white screening of recombinant clones, and a multiple cloning site with numerous unique sites. Plasmids pPB31, pPB33, pPB34, and pPB35 are similar to pPB32 and are described in the text. The promoter (*pmcr*B) and terminator (*tmcr*) of the *Methanococcus voltae* methyl reductase operon regulate expression of the puromycin acetyltransferase (*pac*) gene from *Streptomyces alboniger* in methanoarchaea.

these plates and picked the first colonies to arise from each transformation for subsequent analysis.

Two steps were taken to demonstrate that the mutations conferring PA^r in these clones were within the plasmid-encoded *ileS* gene. First, to ensure that the mutations did not map to the plasmid backbone (such as mutations that might alter the plasmid copy number), the mutagenized *ileS* fragments were subcloned into new shuttle vectors. Second, to ensure that the mutations were not spontaneous mutations in the genomic copy of *ileS*, each new subclone was retransformed into *M. acetivorans* with selection for the vector-encoded puromycin resistance and subsequently screened for PA^r. Nine independent PA^r clones passed this screening test and were characterized in detail. To ensure that all of the mutants analyzed were of independent origin, only a single clone from each mutagenized plasmid pool was examined. The nine plasmids isolated from these clones were designated pPB3, pPB4, pPB5, pPB6, pPB7, pPB22, pPB23, pPB24, and pPB29. The *ileS* alleles present on each plasmid correspond to the plasmid number, i.e., *ileS3*, *ileS4*, *ileS5*, *ileS6*, *ileS7*, *ileS22*, *ileS23*, *ileS24*, and *ileS29*, respectively.

Characterization of *ileS* mutants that confer PA^r. The mutational changes that confer PA^r were determined by DNA sequence analysis of the entire *ileS* gene fragment from each mutant plasmid (Table 1). Surprisingly, six of the nine mutant plasmids carried multiple mutations in *ileS*. Most of the mutations observed were obtained multiple independent times, either alone or in combination with other mutations. Thus, eight of the nine plasmids carry the same G594E mutation, three of the nine carry the G593D mutation, and two of nine carry the A440V mutation. Mutations A482T and G587D were obtained only once. Mutation G594E was obtained as a single mutant three independent times (pPB3, pPB24, and pPB29), while the double mutant G593D/G594E was obtained independently twice (pPB6 and pPB7).

In the plasmids where multiple mutations were observed, it is possible that only one of the changes is responsible for the mutant phenotype. However, the fact that the same few mutations (and in some cases double mutations) were obtained multiple times suggests that most of these mutations contribute individually to the antibiotic resistance phenotype. To address this issue, the individual mutations found in the multiple mutants were separated or combined by subcloning in each case where available restriction endonuclease sites made this possible. Accordingly, plasmid pPB13 (*ileS13*) carries the single mutation A440V, originally found as one of two mutations in plasmid pPB5. Plasmid pPB8 (*ileS8*) carries this mutation in addition to the two mutations found in pPB4, while pPB12 (*ileS12*) combines the two mutations found in pPB6 with the A440V mutation found in pPB5. Interestingly, during the course of these subcloning experiments an *ileS23* triple mutant which is identical to *ileS12* was isolated directly from a mutagenized plasmid pool.

The MIC of pseudomonic acid and the growth rate in medium containing a low, but inhibitory, concentration of pseudomonic acid (35 μg/ml) were determined for *M. acetivorans* transformed with each of the mutant plasmids (Table 1). In general, the effects of the mutations with respect to these phenotypes were additive. Thus, the plasmids pPB8, pPB12, and pPB23, each containing three separate mutations, con-

TABLE 1. Phenotypic characterization of *M. acetivorans* C2A transformed with various *ileS*-carrying plasmids

Plasmid[a]	Presence of mutation:					MIC[b] (μg/ml)	Growth rate[c] (h)
	A440V	A482T	G587D	G593D	G594E		
pJK44	−	−	−	−	−	<35	NG
pPB13	+	−	−	−	−	35	33.3 (1.1)
pPB3	−	−	−	−	+	70	37.1 (8.0)
pPB24	−	−	−	−	+	70	28.0 (0.8)
pPB29	−	−	−	−	+	70	28.3 (0.3)
pPB22	−	−	+	−	−	70	26.6 (1.0)
pPB4	−	+	−	−	+	70	32.9 (6.5)
pPB6	−	−	−	+	+	140	17.8 (0.9)
pPB7	−	−	−	+	+	140	15.8 (0.8)
pPB5	+	−	−	−	+	280	16.5 (1.1)
pPB8	+	+	−	−	+	560	15.1 (0.9)
pPB12	+	−	−	+	+	560	14.9 (1.8)
pPB23	+	−	−	+	+	560	20.5 (0.6)

[a] Plasmid pJK44 carries the wild-type *ileS* gene from *M. barkeri* Fusaro (Fig. 2). Other plasmids are identical to pJK44, except that each carries the mutations indicated.

[b] MICs were determined as described in Materials and Methods in HS-MA medium plus pseudomonic acid added at concentrations of 0, 35, 70, 140, 280, 560, and 1,120 μg/ml. The MIC for *M. acetivorans* C2A was determined to be ca. 12 μg/ml in a different experiment (data not shown).

[c] Generation times were determined as described in Materials and Methods in HS-MA medium plus pseudomonic acid (35 μg/ml). The values shown represent six independent determinations. Standard deviations are in parentheses. NG indicates that growth was not observed. Wild-type *M. acetivorans* C2A grew with a generation time of ca. 6 h in HS-MA medium but did not grow when PA was added to the medium.

ferred PA^r at levels at least twice that conferred by plasmids pPB4, pPB5, pPB6, and pPB7, containing two separate mutations each, and at least four times as high as that conferred by the plasmids pPB3, pPB13, pPB22, pPB24, and pPB29, containing only a single mutation each. One exception is that the A482T mutation does not significantly enhance the level of resistance conferred by the G594E mutation (compare pPB3 with pPB4) but doubles the level of resistance in combination with the mutations A440V and G594E (compare pPB5 with pPB8). Nonetheless, we feel that the A482T mutation in combination with the single mutation G594E must confer a minor advantage for it to have been isolated in our screening procedure.

The growth rate phenotypes were completely consistent with the MIC data. In each case, mutations that conferred higher MICs conferred faster growth rates in medium with pseudomonic acid at 35 μg/ml (Table 1). It should be noted that the growth rate data were somewhat less reproducible than the MIC data. In particular, we noted a difference in the growth rates of strains carrying identical plasmids pPB12 and pPB23. Such differences are not uncommon in the cultivation of these extremely oxygen-sensitive anaerobes, and it was for this reason that we utilized both MIC and growth rate data in the characterization of the *ileS* mutants.

The *M. barkeri* Fusaro *ileS* gene does not recombine with the *M. acetivorans* C2A chromosome. A potential problem with the use of a native gene as a selectable marker is the possibility that recombination between the introduced gene and the resident genomic copy will give rise to antibiotic-resistant clones that are not the desired transformants. Sequence analysis of the internal *ileS* PCR fragments from *M. barkeri* Fusaro and *M. acetivorans* C2A indicates that the genes from the two species are approximately 90% identical at the DNA level (data not shown). Unfortunately, there are no data available regarding the degree of sequence similarity required for homologous recombination among the methanoarchaea. Because of this, we directly tested the ability of *M. barkeri* Fusaro *ileS* to recombine with the *M. acetivorans* gene.

TABLE 2. Recombination of *M. barkeri ileS12* with *M. acetivorans*[a]

Plasmid	Transformation efficiency[b] (CFU/μg of DNA)		
	Pu	PA	Pu + PA
pPB12	2.4×10^5	2.5×10^5	2.3×10^5
pPB18	0	14[c]	0

[a] Recombination between the *ileS* alleles of *M. barkeri* and *M. acetivorans* was examined by transformation of *M. acetivorans* with plasmids carrying the *M. barkeri ileS12* allele that confers PA^r. Plasmid pPB12 is capable of autonomous replication and served as a positive control for transformation frequency. Plasmid pPB18 cannot replicate in *Methanosarcina* and therefore can only give antibiotic-resistant transformants if it recombines with the host chromosome.

[b] Transformation frequencies are averages of two trials with selection on HS-MA agar plus puromycin (Pu), pseudomonic acid (PA), or puromycin plus pseudomonic acid (Pu + PA).

[c] None of the PA^r transformants obtained with pPB18 were resistant to puromycin.

To test for recombination between the two *ileS* genes, we transformed *M. acetivorans* C2A with the plasmids pPB12 and pPB18 (Fig. 2). Both clones carry the PA^r *ileS12* allele and the *pac* gene cassette, which encodes resistance to puromycin in *Methanosarcina*. The plasmids differ in that pPB12 carries the pC2A replicon allowing autonomous replication in *M. acetivorans*, while pPB18 does not. Therefore, transformation with pPB12 should result in antibiotic resistance regardless of the ability to recombine with the host gene; however, because pPB18 is incapable of replication in *M. acetivorans* C2A, antibiotic-resistant transformants are expected only if the plasmid can be integrated into the genome by recombination with the host chromosome. As shown in Table 2, transformation with the self-replicating vector pPB12 results in equal numbers of transformants, regardless of the antibiotic selection used. In contrast, puromycin-resistant transformants were not obtained after transformation with nonreplicating plasmid pPB18. A few PA^r transformants were obtained after transformation with pPB18 (Table 2). These are likely to be spontaneous PA^r mutants because they were not resistant to puromycin, indicating that the plasmid did not integrate into the chromosome. In separate experiments, the frequency of spontaneous PA^r was shown to be $5.5 \times 10^{-9} \pm 1.8 \times 10^{-9}$, consistent with this interpretation (Metcalf, unpublished).

Construction of *Methanosarcina-E. coli* shuttle vectors that confer PA^r. The data indicate that mutant derivatives of *M. barkeri ileS* can be used as selectable markers for PA^r in *M. acetivorans*. Because it confers the highest level of resistance and the fastest growth rate in medium containing pseudomonic acid, we chose to utilize the *ileS12* allele for the construction of *Methanosarcina-E. coli* plasmid shuttle vectors. The plasmids pPB31, pPB32 (Fig. 2), pPB33, pPB34, and pPB35 were constructed as described in Materials and Methods and provide many useful features. Each plasmid is capable of replication in both *E. coli* and *Methanosarcina* utilizing the R6K and pC2A replicons, respectively. Transformants carrying these plasmids can be obtained by selection for ampicillin resistance encoded by the *bla* gene in *E. coli* and in *Methanosarcina* by selection for PA^r encoded by the *ileS12* gene. In addition, the various shuttle vectors possess a variety of restriction sites suitable for cloning and have a *lacZ*α gene to allow blue-white screening for recombinant clones in *E. coli*.

DISCUSSION

The *Methanosarcina-E. coli* shuttle vectors encoding PA^r reported here will be a valuable tool for genetic analysis in *Methanosarcina*. Prior to this report, only a single selectable marker, the *pac* cassette for resistance to puromycin, was available for use in *Methanosarcina* species (29). As a result, it was not previously possible to introduce a second genetic element (either a plasmid or a marker-tagged chromosomal mutation) into a strain that already carried the *pac* cassette. Therefore, standard genetic experiments involving two selectable markers, such as complementation of chromosomal mutations, identification of essential genes, and in vivo identification of genetic regulatory elements, were not possible. With the development of *ileS12*-encoded PA^r selection, such experiments are now possible. Importantly, the finding that plasmid-encoded *ileS* alleles can confer PA^r on otherwise wild-type (PA-sensitive) hosts indicates that the mutant alleles are dominant over the wild-type alleles. Therefore, these *ileS* alleles can be used as selectable markers in genetic experiments using wild-type hosts. Although we expected this to be the case, because in *Staphylococcus* a plasmid-carried *ileS* gene encodes PA^r (13, 33), this finding was by no means certain. For example, the streptomycin sensitivity of the wild-type *rpsL* allele is dominant over streptomycin-resistant *rpsL* alleles in *E. coli* (38).

The utility of the *ileS12* constructs reported here has been proven in two ongoing studies in our laboratory, the results of which will be reported elsewhere. We have used the PA^r shuttle vectors reported here to study hydrogenase function and proline biosynthesis in *Methanosarcina* (W. W. Metcalf, J. K. Zhang, and H. C. Kuettner, unpublished data). Further, we have used the PA^r marker to construct auxotrophic mutants by inserting the *ileS12* gene into cloned *Methanosarcina* proline biosynthesis genes. The disrupted alleles were subsequently crossed onto the *M. acetivorans* chromosome by homologous recombination with selection for PA^r provided by the *ileS12* marker. Ninety percent (108 of 120) of the PA^r recombinants tested were proline auxotrophs. In this regard, it is important to note that we clearly demonstrated that *M. barkeri* Fusaro *ileS* does not recombine with the gene of the closely related species *M. acetivorans*. It is, however, a functional gene in this heterologous host, as shown by its ability to confer PA^r. As such, it is likely that this selectable marker will find use in other *Methanosarcina* species, although the possibility of recombination with the native *ileS* genes in these hosts must be kept in mind.

In addition to their utility in genetic experiments, the mutant *ileS* alleles reported here can further increase our understanding of pseudomonic acid action as an inhibitor of isoleucyl-tRNA synthetase. Pseudomonic acid is hypothesized to act as an analog of the active intermediate isoleucyl-adenylate and to contact residues within the enzyme involved in binding of both ATP and isoleucine (48). As such, the selection for PA^r is quite stringent. Because IleS is required for growth, the mutant protein must remain capable of binding to its natural substrates isoleucine, ATP, and tRNA^{Ile} while no longer binding the antibiotic. At the same time, it must also remain capable of catalyzing tRNA charging. Thus, only subtle changes are expected to be allowed.

Considerable data have accumulated regarding the amino acid residues involved in substrate binding and catalysis by aminoacyl-tRNA synthetases. The known enzymes are divided into two classes of 10 enzymes each. Isoleucyl-tRNA synthetase is a class I aminoacyl-tRNA synthetase since it carries the two signature sequences, HIGH and KMSKS, that are not present in class II aminoacyl-tRNA synthetases (7, 10, 14).

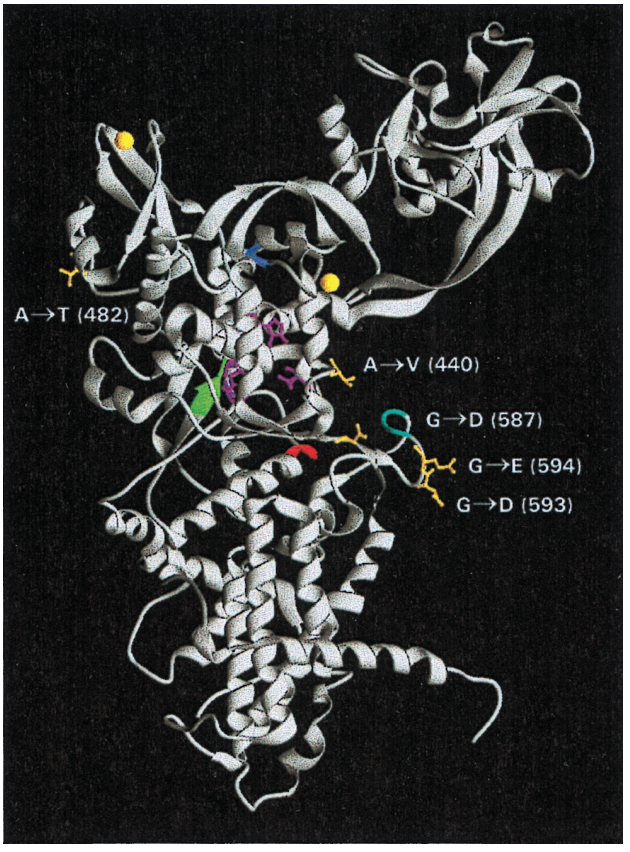

FIG. 3. Modeling of IleS mutations that confer PA^r. The crystal structure of the isoleucyl-tRNA synthetase from *T. thermophilus* (34) was used to identify the homologous positions and spatial locations of amino acid changes in the IleS protein of *M. barkeri* Fusaro that confer PA^r. Side chains of mutated amino acids are in yellow. Bound Zn^{2+} molecules are represented by yellow spheres. The GWD, HIGH, WCISR, and KMSKS consensus sequences of class I aminoacyl-tRNA synthetases are in green, red, blue, and aqua, respectively. The isoleucines binding residues Pro^{46}, Asp^{85}, Trp^{518}, Gln^{554}, and Trp^{558} in *T. thermophilus* IleS are in magenta. The IleS structure was drawn using the program Ribbons 2.0 (9). Coordinates were retrieved from the Protein Data Bank (accession no. 1ILE [5]). See the text for details.

These consensus sequences are indicative of the presence of a nucleotide binding fold, designated the Rossman fold, that contains the active site for ATP binding (10, 14, 18, 37). Structural studies indicate that the amino acid binding site is immediately adjacent to these conserved residues within the active-site pocket of the enzyme (34). Two other consensus sequences have been implicated in IleS activity. The WCISR consensus sequence (amino acids 452 to 456 in *M. barkeri* IleS) has been implicated in the activation of isoleucine. In *E. coli*, *ileS* mutations in this region severely impair isoleucine activation (40). Mutational studies also led to the conclusion that the consensus sequence GWD (amino acids 84 to 86 in *M. barkeri* IleS) is important for isoleucine binding (12, 39). This idea was recently confirmed after determination of the crystal structure of the *Thermus thermophilus* IleS protein containing bound isoleucine indicated that the aspartate residue of the GWD sequence is hydrogen bonded to the NH_3^+ moiety of the amino acid (34).

Analysis of the PA^r mutants isolated in our study, and PA^r mutants of other organisms, supports the hypothesis that pseudomonic acid acts as an analog of the active intermediate isoleucyl-adenylate (48). Using the positions of the analogous

amino acids in the crystal structure of the *T. themophilus* IleS protein, we modeled the approximate positions of the mutated amino acids found in our study (Fig. 3). Four of the five mutations we isolated (A440V, G587D, G593D, and G594E) and one mutation isolated in *E. coli* (48) result in alterations of amino acid residues predicted to lie within with the substrate binding pocket of the enzyme (Fig. 3). The single mutation G594E was present in all but one of the mutated clones we isolated. Further, an identical mutation was found to confer PA^r on *M. thermoautotrophicum* Marburg (21). This mutation is only one amino acid residue upstream of the KMSKS consensus sequence thought to be involved in ATP binding. Similarly, a phenylalanine-to-leucine mutation seven amino acids upstream of the KMSKS consensus sequence was found to confer PA^r on *E. coli* (48). The single mutations G593D and G594E were independently obtained together three times, indicating their importance. The A440V mutation, only 12 amino acids upstream of the WCISR sequence, was also obtained in two independent plasmids and is predicted to lie within the active-site pocket.

The only change that does not appear to modify residues directly associated with those involved in substrate binding is the A482T mutation. As can be seen in Fig. 3, the altered amino acid residue is predicted to lie far from the enzyme active site. As such, it probably exerts its effect by a change in protein conformation; however, it should be noted that these conclusions are based on modeling with an enzyme from a distantly related organism. Therefore, it remains possible that this mutation will actually have a more direct effect in the *Methanosarcina* IleS protein.

Finally, this is the first report of an *ileS* gene sequenced in the genus *Methanosarcina*. Analysis of the sequence of IleS indicates that this protein is closely related to that of other archaea, most closely to that of *A. fulgidus* (Fig. 1). In other studies, IleS has been a valuable phylogenetic marker and has served to support 16S rRNA-based phylogenies, as well as to root the universal tree of life (7). Our finding that *Methanosarcina* IleS fits closely into this previously determined phylogeny is not unexpected, but it may be useful for future phylogenetic studies.

ACKNOWLEDGMENTS

We thank SmithKline Beecham for the kind gift of pseudomonic acid, Kjell Håkansson for assistance with drawing and analysis of the IleS structure, David E. Graham for assistance with the phylogenetic analysis of the IleS protein, and Stanley Maloy for providing strains.

This work was supported by grant MCB-987459 from the National Science Foundation and by a Searle Scholars Award to W.W.M. P.B. was supported in part by National Institutes of Health grant GM 51334.

REFERENCES

1. **Adachi, J., and M. Hasegawa.** 1996. Computer science monographs n 28, MOLPHY version 2.3: programs for molecular phylogenetics based on maximum likelihood. Institute for Statistical Mathematics, Tokyo, Japan.
2. **Argyle, J. L., D. L. Tumbula, and J. A. Leigh.** 1996. Neomycin resistance as a selectable marker in *Methanococcus maripaludis*. Appl. Environ. Microbiol. **62:**4233–4237.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1992. Current protocols in molecular biology, vol. 1 and 2. John Wiley & Sons, Inc., New York, N.Y.
4. **Beckler, G. S., L. A. Hook, and J. N. Reeve.** 1984. Chloramphenicol acetyltransferase should not provide methanogens with resistance to chloramphenicol. Appl. Environ. Microbiol. **47:**868–869.
5. **Bernstein, F. C., T. F. Koetzle, G. J. B. Williams, E. F. Meyer, M. D. Brice, J. R. Rodgers, O. Kennard, T. Shimanouchi, and M. Tasumi.** 1977. The Protein Data Bank: a computer-based archival file for macromolecular structures. J. Mol. Biol. **112:**535–542.
6. **Bock, A., and O. Kandler.** 1985. Antibiotic sensitivity of archaebacteria, p. 525–544. *In* C. R. Woese and R. S. Wolfe (ed.), Archaebacteria, vol. 8. Academic Press, Inc., New York, N.Y.
7. **Brown, J. R., and W. F. Doolittle.** 1995. Root of the universal tree of life based on ancient aminoacyl-tRNA synthetase gene duplications. Proc. Natl. Acad. Sci. USA **92:**2441–2445.
8. **Bult, C. J., O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann, G. G. Sutton, J. A. Blake, L. M. FitzGerald, R. A. Clayton, J. D. Gocayne, A. R. Kerlavage, B. A. Dougherty, J. F. Tomb, M. D. Adams, C. I. Reich, R. Overbeek, E. F. Kirkness, K. G. Weinstock, J. M. Merrick, A. Glodek, J. L. Scott, N. S. M. Geoghagen, and J. C. Venter.** 1996. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science **273:**1058–1073.
9. **Carson, M.** 1991. Ribbons 2.0. J. Appl. Crystallogr. **24:**958–961.
10. **Carter, C. W.** 1993. Cognition, mechanism, and evolutionary relationships in aminoacyl-tRNA synthetases. Annu. Rev. Biochem. **62:**715–748.
11. **Chambers, S. P., S. E. Prior, D. A. Barstow, and N. P. Minton.** 1988. The pMTL nic-cloning vectors. I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. Gene **68:**139–149.
12. **Clarke, N. D., D. C. Lien, and P. Schimmel.** 1988. Evidence from cassette mutagenesis for a structure-function motif in a protein of unknown structure. Science **240:**521–523.
13. **Dyke, K. G., S. P. Curnock, M. Golding, and W. C. Noble.** 1991. Cloning of the gene conferring resistance to mupirocin in *Staphylococcus aureus*. FEMS Microbiol. Lett. **61:**195–198.
14. **Eriani, G., M. Delaure, O. Poch, J. Gangloff, and D. Moras.** 1990. Partition of tRNA synthetases into two classes based on mutually exclusive sets of sequence motifs. Nature **347:**203–206.
15. **Fuller, A. T., G. Mellows, M. Woolford, G. T. Banks, K. D. Barrow, and E. B. Chain.** 1971. Pseudomonic acid: an antibiotic produced by *Pseudomonas fluorescens*. Nature **234:**416–417.
16. **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.
17. **Hedderich, R., J. Koch, D. Linder, and R. K. Thauer.** 1994. The heterodisulfide reductase from *Methanobacterium thermoautotrophicum* contains sequence motifs characteristic of pyridine-nucleotide-dependent thioredoxin reductases. Eur. J. Biochem. **225:**253–261.
18. **Hou, Y. M., K. Shiba, C. Mottes, and P. Schimmel.** 1991. Sequence determining and modeling of structural motifs for the smallest monomeric aminoacyl transfer RNA synthetase. Proc. Natl. Acad. Sci. USA **88:**976–980.
19. **Hughes, J., and G. Mellows.** 1978. Inhibition of isoleucyl-transfer ribonucleic acid synthetase in *Escherichia coli* by pseudomonic acid. Biochem. J. **176:** 305–318.
20. **Hughes, J., G. Mellows, and S. Soughton.** 1980. How does *Pseudomonas fluorescens*, the producing organism of the antibiotic pseudomonic acid A, avoid suicide? FEBS Lett. **122:**322–324.
21. **Jenal, U., T. Rechsteiner, P. Y. Tan, E. Buhlmann, L. Meile, and T. Leisinger.** 1991. Isoleucyl-tRNA synthetase of *Methanobacterium thermoautotrophicum* Marburg. Cloning of the gene, nucleotide sequence, and localization of a base change conferring resistance to pseudomonic acid. J. Biol. Chem. **266:**10570–10577.
22. **Jermiin, L. S., G. J. Olsen, K. L. Mengersen, and Easteal.** 1997. Majority-rule consensus of phylogenetic trees obtained by maximum-likelihood analysis. Mol. Biol. Evol. **14:**1296–1302.
23. **Jones, W. J., W. B. Whitman, R. D. Fields, and R. S. Wolfe.** 1983. Growth and plating efficiency of methanococci on agar media. Appl. Environ. Microbiol. **46:**220–226.
24. **Klenk, H. P., R. A. Clayton, J. F. Tomb, O. White, K. E. Nelson, K. A. Ketchum, R. J. Dodson, M. Gwinn, E. K. Hickey, J. D. Peterson, D. L. Richardson, A. R. Kerlavage, D. E. Graham, N. C. Kyrpides, R. D. Fleischmann, J. Quackenbush, N. H. Lee, G. G. Sutton, S. Gill, E. F. Kirkness, B. A. Dougherty, K. McKenney, M. D. Adams, B. Loftus, J. C. Venter, et al.** 1997. The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. Nature **390:**364–370.
25. **Maloy, S. R.** 1990. Hydroxylamine mutagenesis, p. 50–54. *In* S. R. Maloy (ed.), Experimental techniques in bacterial genetics. Jones and Bartlett, Boston, Mass.
26. **Meile, L., P. Abendschein, and T. Leisinger.** 1990. Transduction in the archaebacterium *Methanobacterium thermoautotrophicum* Marburg. J. Bacteriol. **172:**3507–3508.
27. **Metcalf, W. W.** 1999. Genetic analysis in the domain Archaea Methods Microbiol. **29:**277–326.
28. **Metcalf, W. W., W. Jiang, L. L. Daniels, S. K. Kim, A. Haldimann, and B. L. Wanner.** 1996. Conditionally replicative and conjugative plasmids carrying *lacZ*α for cloning, mutagenesis, and allele replacement in bacteria. Plasmid **35:**1–13.
29. **Metcalf, W. W., J. K. Zhang, E. Apolinario, K. R. Sowers, and R. S. Wolfe.** 1997. A genetic system for Archaea of the genus *Methanosarcina*: liposome-mediated transformation and construction of shuttle vectors. Proc. Natl. Acad. Sci. USA **94:**2626–2631.
30. **Metcalf, W. W., J. K. Zhang, X. Shi, and R. S. Wolfe.** 1996. Molecular, genetic, and biochemical characterization of the *serC* gene of *Methanosarcina barkeri* Fusaro. J. Bacteriol. **178:**5797–5802.

31. **Metcalf, W. W., J. K. Zhang, and R. S. Wolfe.** 1998. An anaerobic, intrachamber incubator for growth of *Methanosarcina* spp. on methanol-containing solid media. Appl. Environ. Microbiol. **64:**768–770.
32. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
33. **Morton, T. M., J. L. Johnston, J. Patterson, and G. L. Archer.** 1995. Characterization of a conjugative staphylococcal mupirocin resistance plasmid. Antimicrob. Agents Chemother. **39:**1272–1280.
34. **Nureki, O., G. Vassylyev Dmitry, M. Tateno, A. Shimada, T. Nakama, S. Fukai, M. Konno, L. Hendrickson Tamara, P. Schimmel, and S. Yokoyama.** 1998. Enzyme structure with two catalytic sites for double-sieve selection of substrate. Science **280:**578–582.
35. **Pfeiffer, M., H. Bestgen, A. Burger, and A. Klein.** 1998. The *vhuU* gene encoding a small subunit of a selenium-containing [NiFe]$^-$ hydrogenase in *Methanococcus voltae* appears to be essential for the cell. Arch. Microbiol. **170:**418–426.
36. **Possot, O., P. Gernhardt, A. Klein, and L. Sibold.** 1988. Analysis of drug resistance in the archaebacterium *Methanococcus voltae* with respect to potential use in genetic engineering. Appl. Environ. Microbiol. **54:**734–740.
37. **Rossman, M. G., A. Liljas, C. I. Branden, and L. J. Banaszak.** 1975. Evolutionary and structural relationships among dehydrogenases, 3rd ed., vol. 11. Academic Press, Orlando, Fla.
38. **Russell, C. B., and F. W. Dahlquist.** 1989. Exchange of chromosomal and plasmid alleles in *Escherichia coli* by selection for loss of a dominant antibiotic sensitivity marker. J. Bacteriol. **171:**2614–2618.
39. **Schmidt, E., and P. Schimmel.** 1993. Dominant lethality by expression of a catalytically inactive class I tRNA synthetase. Proc. Natl. Acad. Sci. USA **90:**6919–6923.
40. **Schmidt, E., and P. Schimmel.** 1995. Residues in a class I tRNA synthetase which determine selectivity of amino acid recognition in the context of tRNA. Biochemistry **34:**11204–11210.
41. **Smith, D. R., L. A. Doucette-Stamm, C. Deloughery, H. Lee, J. Dubois, T. Aldredge, R. Bashirzadeh, D. Blakely, R. Cook, K. Gilbert, D. Harrison, L. Hoang, P. Keagle, W. Lumm, B. Pothier, D. Qiu, R. Spadafora, R. Vicaire, Y. Wang, J. Wierzbowski, R. Gibson, N. Jiwani, A. Caruso, D. Bush, et al.** 1997. Complete genome sequence of *Methanobacterium thermoautotrophicum* ΔH: functional analysis and comparative genomics. J. Bacteriol. **179:** 7135–7155.
42. **Sowers, K. R., J. Boone, and R. P. Gunsalus.** 1993. Disaggregation of *Methanosarcina* spp. and growth as single cells at elevated osmolarity. Appl. Environ. Microbiol. **59:**3832–3839.
43. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
44. **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.
45. **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.
46. **Wanner, B. L.** 1986. Novel regulatory mutants of the phosphate regulon in *Escherichia coli* K-12. J. Mol. Biol. **191:**39–58.
47. **Ward, A., and D. M. Campoli-Richards.** 1986. Mupirocin: a review of its antibacterial activity, pharmacokinetic properties and therapeutic use. Drugs **32:**425–444.
48. **Yanagisawa, T., J. T. Lee, H. C. Wu, and M. Kawakami.** 1994. Relationship of protein structure of isoleucyl-tRNA synthetase with pseudomonic acid resistance of *Escherichia coli*: a proposed mode of action of pseudomonic acid as an inhibitor of isoleucyl-tRNA synthetase. J. Biol. Chem. **269:**24304–24309.