JOURNAL OF BACTERIOLOGY, Feb. 2005, p. 972–979 Vol. 187, No. 3
0021-9193/05/$08.00+0 doi:10.1128/JB.187.3.972–979.2005

Markerless Mutagenesis in *Methanococcus maripaludis* Demonstrates Roles for Alanine Dehydrogenase, Alanine Racemase, and Alanine Permease

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Received 27 May 2004/Accepted 29 October 2004

Among the archaea, *Methanococcus maripaludis* has the unusual ability to use L- or D-alanine as a nitrogen source. To understand how this occurs, we tested the roles of three adjacent genes encoding homologs of alanine dehydrogenase, alanine racemase, and alanine permease. To produce mutations in these genes, we devised a method for markerless mutagenesis that builds on previously established genetic tools for *M. maripaludis*. The technique uses a negative selection strategy that takes advantage of the ability of the *M. maripaludis hpt* gene encoding hypoxanthine phosphoribosyltransferase to confer sensitivity to the base analog 8-azahypoxanthine. In addition, we developed a negative selection method to stably incorporate constructs into the genome at the site of the *upt* gene encoding uracil phosphoribosyltransferase. Mutants with in-frame deletion mutations in the genes for alanine dehydrogenase and alanine permease lost the ability to grow on either isomer of alanine, while a mutant with an in-frame deletion mutation in the gene for alanine racemase lost only the ability to grow on D-alanine. The wild-type gene for alanine dehydrogenase, incorporated into the *upt* site, complemented the alanine dehydrogenase mutation. Hence, the permease is required for the transport of either isomer, the dehydrogenase is specific for the L isomer, and the racemase converts the D isomer to the L isomer. Phylogenetic analysis indicated that all three genes had been acquired by lateral gene transfer from the low-moles-percent G+C gram-positive bacteria.

Methanococcus maripaludis is a methanogenic archaeon isolated from salt marsh sediments (12). A hydrogenotrophic methanogen, *M. maripaludis* generates methane from hydrogen and carbon dioxide or formate. Relatively rapid growth (13), genetic tools (25), and a complete genome sequence (11) contribute to the utility of *M. maripaludis* as a laboratory model. Numerous mutants have been generated by positive selection for homologous recombination leading to genetic insertion or gene replacement (e.g., see references 17 and 20). However, an efficient method for the generation of markerless mutations has not previously been available.

Markerless mutations, including in-frame deletions and point mutations, have several advantages. Genes of interest are altered or disrupted with minimal perturbation of surrounding genes. The potential for polar effects caused by insertions is avoided. Moreover, selectable markers used during the introduction of the mutations are removed, allowing their repeated use in subsequent manipulations.

Efficient generation of markerless mutations requires both selection and counterselection, the latter with a negative selectable marker. Typically, markers for both positive and negative selection are provided on a plasmid vector, into which a mutant construct is cloned. First, positive selection is used to obtain homologous recombination of the recombinant plasmid into the genome, creating a merodiploid. Negative selection is then used to force removal of the vector by a second homologous recombination event. The result is either the wild-type allele or the desired mutation, depending on whether the two recombination events occur on the same side or different sides of the mutation. The mutations, which are present in up to half of the isolates, are easily identified by rapid screening.

Negative selection is routine in several species. In all cases the negative selectable marker encodes an enzyme that, when presented with a particular substrate, produces a toxic product. The *Bacillus subtilis sacB* gene encoding levansucrase confers sucrose sensitivity when expressed in *Escherichia coli* and other gram-negative bacteria (22). In yeast, the *ura3* gene encoding orotidine-5′-phosphate decarboxylase mediates sensitivity to 5-fluoroorotic acid (5). Similarly, *pyrF* encoding orotidine-5′-phosphate decarboxylase and *pyrE* encoding orotate phosphoribosyltransferase make *Halobacterium* sp. and *Haloferax volcanii*, respectively, sensitive to 5-fluoroorotic acid (3, 19, 26). Recently, *hpt* encoding hypoxanthine phosphoribosyltransferase (Hpt) and conferring sensitivity to the base analog 8-aza-2,6-diaminopurine was used for negative selection to produce markerless mutations in *Methanosarcina acetivorans* (21).

The susceptibility of *Methanococcus voltae* (a close relative of *M. maripaludis*) to base analogs was reported by Bowen and Whitman (7). *M. maripaludis* also proved susceptible to base analogs, which were used in a strategy to enrich for auxotrophic mutants (14). These results suggested the presence of nucleotide salvage pathways, which presumably could incorporate base analogs to form toxic nucleotides. The presence of guanine phosphoribosyltransferase and Hpt activities was confirmed in *M. voltae*, and spontaneous mutants resistant to base analogs lacked these activities (6).

The above observations suggested that phosphoribosyltransferase genes should serve as negative selectable markers in *M. maripaludis*, since their presence would confer sensitivity to base analogs. Here we report the development of Hpt- and

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Features	Source or reference
Strains		
S2 (formerly LL)	Wild-type *M. maripaludis*	28
Mm900	S2 Δ*hpt*	This study
Mm1002	Mm900 Δ*ald*	This study
Mm1018	Mm900 Δ*agcS*	This study
Mm1031	Mm900 Δ*alr*	This study
Mm1068	Mm1002 Δ*upt*::*ald*	This study
Plasmids		
pWLG40NZ-R	Replicative plasmid in *M. maripaludis* and *E. coli* with *hmv* promoter and Neo^r cassette	17
pBCPrt2.0	*hmv* promoter-*hpt* fusion in pWLG40NZ-R	This study
pCR2.1TOPO	Amp^r Kan^r cloning vector	Invitrogen
pCRPrtNeo	*hmv* promoter-*hpt* fusion + Neo^r cassette in pCR2.1TOPO	This study
pCR2.1U	*upt* + flanking DNA in pCR2.1TOPO	This study
pCR2.1U1	In-frame deletion of *upt*	This study
pCR2.1	Amp^r Kan^r cloning vector	Invitrogen
pCR2.1U1Δmcs	pCR2.1U1 without multiple cloning sites in pCR2.1	This study
pBLPrt	*hmv-hpt-neo* cassette from pBCPrt2.0 in pCR2.1U1Δmcs	This study
pBIPrtnatAld	*ald* between *upt*-flanking sequences in pBLPrt	This study
pCR2.1A	*hpt* + flanking DNA in pCR2.1TOPO	This study
pCR2.1A1	In-frame deletion of *hpt*	This study
pBCAla1.1	*ald* + flanking DNA in pCR2.1TOPO	This study
pBCΔAla	In-frame deletion of *ald*	This study
pCRPTNΔAla	In-frame deletion of *ald* in pCRPrtNeo	This study
pBCAlaPer	*agcS* + flanking DNA in pCR2.1TOPO	This study
pBCΔAlaPer	In-frame deletion of *agcS*	This study
pCRPrtΔAlaPer	In-frame deletion of *agcS* in pCRPrtNeo	This study
pBCΔAlaRace	In-frame deletion of *alr* in pCR2.1TOPO	This study
pCRPrtΔAlaRace	In-frame deletion of *alr* in pCRPrtNeo	This study

Upt (uracil phosphoribosyltransferase)-based negative selection systems for *M. maripaludis*. We used the Hpt system to produce markerless in-frame deletion mutations in three genes, *ald* encoding alanine dehydrogenase, *alr* encoding alanine racemase, and *agcS* encoding a sodium-alanine symporter (alanine permease). We also used Hpt together with Upt to incorporate wild-type *ald*, which complemented Δ*ald*. *M. maripaludis* is unusual in its ability to use alanine as a nitrogen source (27), and among the archaea whose genome sequences are known, the three genes are uniquely present in *M. maripaludis*. The mutations generated here allowed us to determine the role of each gene in the use of both L- and D-alanine. We also used phylogenetic analysis to investigate the role of lateral gene transfer in the acquisition of these genes.

MATERIALS AND METHODS

Strains, cultures, and growth conditions. *M. maripaludis* strain S2 (wild type, formerly LL) and its derivatives were grown with strict anaerobic techniques at 37°C with H_2-CO_2 (80:20) as previously described (2). For plating, we modified McC medium (28) by replacing yeast extract with Casamino Acids (Difco), since yeast extract reduced the sensitivity of *M. maripaludis* to base analogs; the modified medium was designated McCas. For broth culture we used McCas or nitrogen-free minimal medium (4) supplemented with the appropriate nitrogen source from sterile, anaerobic stocks. Neomycin sulfate (1.0 mg/ml of broth and 0.5 mg/ml of agar) (1), 8-azahypoxanthine (0.25 mg/ml), and 6-azauracil (0.25 mg/ml) were added from sterile, anaerobic stocks as needed. The 8-azahypoxanthine and 6-azauracil stocks were 10 mg/ml in 1 M NaOH. *E. coli* strains were grown at 37°C. Kanamycin (0.045 mg/ml) and ampicillin (0.1 mg/ml) were added as needed.

Plasmid construction. All of the plasmids and strains used in this study are listed in Table 1. pCRPrtNeo was constructed as follows. The *hpt* gene was PCR amplified from fosmid DNA (from a bank of S2 genomic DNA) with Herculase polymerase (Stratagene) and primers Prtnsi1F and Prtbgl2R (the sequences of the primer used are listed in Table 2), cut with NsiI and BglII, and ligated into NsiI/BglII-cut pWLG40NZ-R. The result was pBCPrt2.0, containing *hpt* fused to the constitutive *hmv* promoter and a Neo^r cassette. The *hmv* promoter-*hpt* fusion with the Neo^r cassette was amplified with primers pbcprt2.0avr2F and pbcprt2.0afl2R and TA cloned into pCR2.1TOPO (Invitrogen), resulting in pCRPrtNeo.

pBLPrt and pBlPrtnatAld were constructed as follows. The *upt* gene with flanking DNA was amplified from fosmid DNA with primers uprt3 and uprt4 and TOPO cloned into pCR2.1TOPO to make pCR2.1U. An in-frame deletion of *upt* was then generated by amplifying around pCR2.1U with primers uprtdelstopAsc1 and uprtdelstopAsc1 and EasyA polymerase (Stratagene), digesting the product with Asc1, and ligating the ends to make pCR2.1U1. Multiple cloning sites were eliminated by amplifying pCR2.1U1 with EasyA polymerase and primers uprt3-XbaI and uprt4-KpnI, digesting the product with XbaI and KpnI, and ligating it into XbaI/KpnI-digested pCR2.1 to produce pCR2.1U1Δmcs. The *hmv-hpt-neo* cassette was then amplified from pBCPrt2.0 with primers pbcPrt2.0-XbaIR and pbcPrt2.0XbaIF, digested with XbaI, and ligated into XbaI-digested pCR2.1U1Δmcs to create pBlPrt. The *ald* gene (including 120 bases upstream) was amplified with EasyA and primers aldnatF and 100-1rev, digested with AscI, and ligated into AscI-digested pBlPrt to make pBlPrtnatAld.

An in-frame deletion in *hpt* was constructed as follows. The *hpt* gene with 949 bp of upstream DNA and 1,358 bp of downstream DNA was amplified by PCR from genomic DNA of *M. maripaludis* S2 with primers Aprt1 and Aprt2. The product was TA cloned into pCR2.1TOPO to yield pCR2.1A. An in-frame deletion was then generated by amplifying outward from the edge of the gene with primers Aprtdelstart and Aprtdelstop, digesting the product with BsiWI, and ligating the ends, yielding pCR2.1A1.

Similarly, an in-frame deletion in *ald* was constructed with primers Aladehydrogspe12F and Aladehydrognot12R to amplify the gene from fosmid DNA and primers Δalaasc1R and Δalaasc1L and AscI to make the deletion. The resulting construct coded for a 10-amino-acid peptide flanked by 1,002 bp of upstream DNA and 1,010 bp of downstream DNA. The deletion construct was then recloned by digestion with BamHI and ligation into BamHI-cut pCRPrtNeo to yield pCRPTNΔAla.

An in-frame deletion in *agcS* was made in the same way, with fosmid DNA, primers AlaninepermeaseF and AlaninepermeaseR, primers AlanineΔperasc1start and AlanineΔperasc1stop, and AscI. The resulting construct coded for a three-amino-acid peptide flanked by 428 and 701 bp upstream and downstream, respectively. The deletion construct was then recloned by amplification with prim-

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)	Restriction site
Aprt1	CCTCGAGCTTGAAAATACGCCGCTCTGG	XhoI
Aprt2	CGGATCCGAGGGGTGGCGCCTTAAAAG	BamHI
Aprtdelstart	GGCGTACGCAATAATTTGCTCATATAATT	BsiWI
Aprtdelstop	GCCGTACGGAATAATTTTCAGGTGAAATT	BsiWI
Prtnsi1F	CCAATGCATGAGCAAATTATTGGAAGAATC	NsiI
Prtbgl2R	GGAAGATCTTTATTCTAAAATGTTTACTTTTCC	BglII
Pbcprt2.0avr2F	TCCGGACCTAGGTTGCATATATCATTGTTAGACC	AvrII
Pbcprt2.0afl2R	GGATCCCTTAAGAAGGATCTTCACCTAGATCC	AflII
Aladehydrogspe12F	CAACTAGTGGATATCTGCATATAGGTACGG	SpeI
Aladehydrognot12R	CTGTACGAATGCGGCCGCGCAGATTCTGCCAACAACAGG	NotI
Δalaasc1R	TTGGCGCGCCAAAGCACACGACCTTCCATAC	AscI
Δalaasc1L	TTGGCGCGCCTCCAATTAGCATGTCAATCCC	AscI
AlaninepermeaseF	TGTTGTTGGCAGAATCTGC	
AlaninepermeaseR	TGATACTACACCGTATGAAGG	
AlanineΔperasc1start	GGCGCGCCCATTTTTCCACTCCAAATTT	AscI
AlanineΔperasc1stop	GGCGCGCCTTAAATTAACAATTAATTACC	AscI
Alanineper-xba1R	GCTCTAGATGATACTACACCGTATGAAGG	XbaI
Alanineper-xba1F	GCTCTAGATGTTGTTGGCAGAATCTGC	XbaI
AlanineracemaseF	TAGCTATCACACCAGCAGG	
AlanineracemaseR	GTCCACCAAGAACGTATGC	
Racexba1-1F	TCATTCTAGATCCACCAAGAACGTATGC	XbaI
Raceasc1-1R	TTGGCGCGCCGTATCTGCAAGAGTTCCAAGA	AscI
Racexba1-start-del	CTAGTCTAGAACTCGCTTACTTAGACG	XbaI
Raceasc1-start-del	TTGGCGCGCCCTTTTACTACTGCCATTACC	AscI
UPRT3	GGATATCGTGCTTTGATTATAGGCGGGG	
UPRT4	GGAATTCCTAAACCTCCCCAGAAAAGAC	
UprtΔstartAscI	GGCGCGCCGAATAAGCATTTATATGTTTC	AscI
UprtΔstopAscI	GGCGCGCCTCATAATTTACCCTCTAAATCC	AscI
Uprt3-XbaI	GCTCTAGAGATATCGTGCTTTGATTATAGGCGGG	XbaI
Uprt4-KpnI	GCGGTACCGAATTCCTAAACCTCCCCAGAAAAGAC	KpnI
pbcPrt2.0-XbaIR	TAGCTCTAGAAAGGATCTTCACCTAGATCC	XbaI
pbcPrt2.0-XbaIF	TAGCTCTAGATTGCATATATCATTGTTAGACC	XbaI
aldnatF	GGGGCGCGCCAATTCTTGAGTATATAAATGAAGTC	AscI
100-1rev	AAGGCGCGCCTTATTCATTTAAAGCTTTTTC	AscI

ers Alanineper-xba1R and Alanineper-xba1F, digestion with XbaI, and ligation into XbaI-cut pCRPrtNeo.

An in-frame deletion in *alr* was made by first PCR amplifying separately both flanking sequences from fosmid DNA with primers Racexba1-1F and Raceasc1-1R and primers Racexba1-start-del and Raceasc1-start-del. Both PCR products were digested with AscI, ligated together, and PCR amplified with primers Racexba1-1F and Raceasc1-start-del. The product was TA cloned into pCR2.1TOPO. The resulting construct coded for a 51-amino-acid peptide flanked by 570 and 498 bp upstream and downstream, respectively. The deletion construct was then recloned by amplification with primers Racexba1-1F and Racexba1-start-del, digestion with XbaI, and ligation into XbaI-cut pCRPrtNeo.

All of the plasmid regions obtained by PCR were sequenced to verify that no errors were introduced. Sequencing was done at the Department of Biochemistry sequencing center, University of Washington.

Strain construction. Strain Mm900 (S2 Δ*hpt*) was made by transforming (24) S2 with pCR2.1A1 DNA and plating it onto McCas agar containing 8-azahypoxanthine. Colonies were screened for the presence of the deletion with primers Aprt1 and Aprt2. Mm1002, Mm1018, and Mm1031 were constructed as follows. Mm900 was transformed with pCRPTNΔAla, pCRPrtΔAlaPer, and pCRPrtΔAlaRace, respectively, and plated onto McCas with neomycin. One Neo^r colony was picked and grown overnight in McCas broth with neomycin to eliminate any remaining neomycin-sensitive cells. This culture (0.05 ml) was used to inoculate McCas broth (5 ml) without neomycin to allow a second recombination event to remove the vector. Finally, this culture was plated (0.1 ml) onto McCas agar with 8-azahypoxanthine. Strain Mm1068 (Mm1002 Δ*upt*::*ald*) was made in the same way with pBlPrtnatAld and Mm1002 and selection with 8-azahypoxantine and 6-azauracil. All strains were streak purified and verified by Southern blotting with probes derived from cloned genes.

Southern analysis. Southern blot assays were performed with a digoxigenin-labeled probe in accordance with the manufacturer's (Roche, Mannheim, Germany) recommendations with the following modifications. Prehybridization mixtures were incubated for 2 h at 55°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% Sarkosyl–0.02% sodium dodecyl sulfate–0.5% blocking reagent (Roche). Buffer was replaced with the same solution containing 10 ng of probe per ml, and hybridization mixtures were incubated overnight at 55°C. Blots were washed four times for 15 min each time in a solution of 0.3× SSC and 1% sodium dodecyl sulfate at 55°C, rinsed for 1 min at room temperature in TRIS solution (100 mM Tris-HCl [pH 7.5], 150 mM NaCl), and then incubated for 45 min in TRIS solution plus 0.5% blocking reagent. Blots were rinsed for 1 min in TRIS solution and then incubated for 45 min at room temperature in TRIS buffer plus 0.5% blocking reagent containing a 1:5,000 dilution of anti-digoxigenin antibody. Blots were shaken twice for 10 min each time in TRIS solution, incubated in detection buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM $MgCl_2$) for 1 min, and visualized with the BCIP/NBT-Purple Liquid Substrate System for Membranes (Sigma).

Growth curves. Overnight cultures were grown in McCas broth, and 0.04 ml was used to inoculate tubes of N-free minimal medium supplemented with the appropriate nitrogen source. Optical density at 600 nm was measured against a water blank.

Gene sequences and phylogenetic analysis. The complete genome sequence of *M. maripaludis* is deposited at EMBL/GenBank/DDBJ under accession number BX950229. The open reading frame numbers of *M. maripaludis* genes are as follows: *hpt*, Mmp0145; *ald*, Mmp1513; *alr*, Mmp1512; *agcS*, Mmp1511; *upt*, Mmp0680. Homologs were found with Blastp in NCBI-Blast2 at the European Bioinformatics Institute (http://www.ebi.ac.uk/services/index.html). DB Clustal was then run on selected homologs, and the results were pasted into Clustal W for final alignment. Gaps were edited with Genedoc (http://www.psc.edu/biomed/genedoc/) (18), phylogenic analyses were done with the Phylip package (http://evolution.genetics.washington.edu/phylip.html), and tree diagrams were obtained with Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

RESULTS

***M. maripaludis* can use L- or D-alanine as a nitrogen source.** Among archaea, *M. maripaludis* has the unusual ability to use

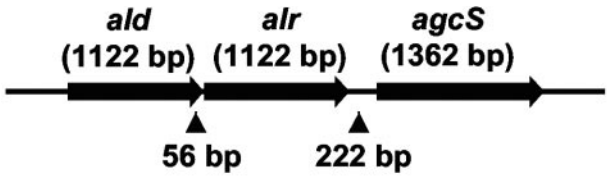

FIG. 1. Alanine utilization loci of *M. maripaludis*. *ald* encodes alanine dehydrogenase, *alr* encodes alanine racemase, and *agcS* encodes alanine-sodium symport. The gene cluster is flanked by *tyrA* (prephenate dehydrogenase, 315 bp upstream on the opposite strand) and *gatA* (aspartyl-tRNA^{asn}/glutamyl-tRNA^{gln} amidotransferase subunit A, 178 bp downstream on the same strand).

L-alanine as a nitrogen source (27). We found that D-alanine served as a nitrogen source as well (see Fig. 4). The recently completed genome sequence of *M. maripaludis* (11) reveals the presence of adjacent genes encoding homologs of alanine dehydrogenase, alanine racemase, and alanine permease (sodium-alanine symporter) (Fig. 1). We hypothesized that alanine dehydrogenase could produce ammonia from alanine, which could be assimilated. If the alanine dehydrogenase had a stereospecificity for the L isomer, then the alanine racemase could provide the additional ability to use D-alanine. The permease could facilitate the import of either isomer. We devised a system for markerless mutagenesis of *M. maripaludis* and made an in-frame deletion mutation in each of the three genes hypothetically involved in alanine utilization.

In-frame deletion of *hpt* removes sensitivity to the analog 8-azahypoxanthine. The previously observed sensitivity of *M. maripaludis* to 8-azahypoxanthine could be attributed to Hpt encoded by the *hpt* gene (6, 7, 14). We constructed a plasmid containing an in-frame deletion in the *hpt* gene, flanked by 0.9 kb of upstream DNA and 1.4 kb of downstream DNA. Transforming wild-type strain S2 with the plasmid DNA and selecting with 8-azahypoxanthine resulted in replacement of wild-type *hpt* with Δ*hpt* in a single step. Twelve colonies were screened by PCR. Eight yielded a product of the expected size for the deletion, while the remaining four retained the wild-type gene. One transformant was screened by Southern blotting and contained the deletion, no wild-type *hpt*, and no plasmid backbone. The mutant, S2 Δ*hpt*, was designated Mm900. When Mm900 was plated in the presence of 8-azahypoxanthine, 4.6% of the viable cells yielded colonies whereas S2 yielded only 1 in 10^{-6}. Hence, deletion of *hpt* substantially removed sensitivity to 8-azahypoxanthine, and Mm900 is a suitable background strain for making markerless mutations. Mm900 had the same plating efficiency as S2 and had no noticeable growth deficiency.

Markerless mutagenesis. To devise a system for markerless mutagenesis in the Mm900 background, we constructed pCRPrtNeo (Fig. 2), a plasmid containing a Neo^r marker for positive selection in *M. maripaludis*, *hpt* for negative selection, and a cloning site. pCRPrtNeo contains no origin for replication in *M. maripaludis*. An in-frame deletion of each gene hypothesized to be involved in alanine utilization was then cloned with flanking DNA into pCRPrtNeo. We used two different cloning methods to obtain the in-frame deletions. One method involved PCR amplification outward from the ends of the gene to be deleted and around the entire vector, followed by end digestion, ligation, cloning, and finally recloning into pCRPrtNeo (see Materials and Methods). In the second, more facile method, we separately amplified two fragments containing each end of the gene with flanking DNA, digested their ends, ligated them together, and PCR amplified the product without any cloning step until the final insertion into pCRPrtNeo.

Each deletion construct was transformed into Δ*hpt* mutant strain Mm900 with selection for neomycin resistance. In each case a single homologous recombination mediated by DNA flanking the presumptive alanine gene presumably produced a merodiploid that contained wild-type and mutant gene alleles separated by vector DNA. Using a single neomycin-resistant colony, we grew a broth culture in the presence of neomycin and used that culture to inoculate a second broth culture, this time without neomycin, that we grew overnight. We predicted that a second recombination event during growth without selection should remove the vector backbone, resulting in loss of both Neo^r and *hpt*, and the products would be selected by subsequent growth on agar medium containing 8-azahypoxanthine. Vector loss should be accompanied by reversion to the wild-type allele of the alanine gene or the generation of a markerless mutation in the alanine gene, depending on whether the first and second recombination events occurred on the same side or different sides of the DNA flanking the deletion. In either case, 8-azahypoxanthine resistance would result.

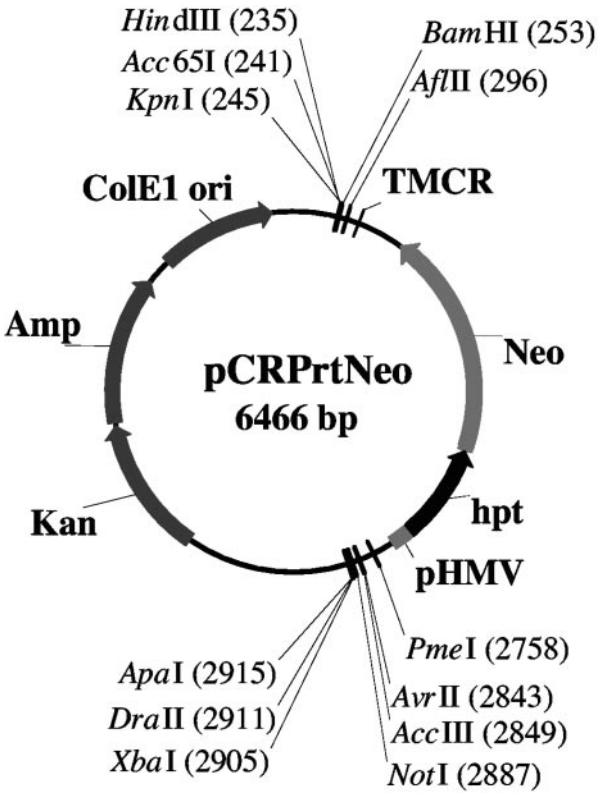

FIG. 2. Plasmid pCRPrtNeo. DNA to be introduced into *M. maripaludis* can be inserted into multiple cloning sites. Markers for positive (Neo) and negative (hpt) selection in *M. maripaludis* are present. ColE1 ori, ColE1 origin of replication; Amp, ampicillin resistance in *E. coli*; Kan, kanamycin resistance in *E. coli*; Neo, neomycin resistance in *M. maripaludis*; hpt, *M. maripaludis hpt* gene; pHMV, histone gene promoter from *M. voltae* (9); TMCR, *mcr* gene terminator from *M. voltae* (10). All of the restriction sites shown are unique.

TABLE 3. Frequencies of genetic events leading to markerless mutations

Strain	Frequency of 8-azahypoxanthine resistance[a]	No. of deletion mutants/total no. of colonies scored
S2	2.1×10^{-6}[b]	
Mm900	4.6×10^{-2}	
Δ*ald* merodiploid	3.5×10^{-4}	8/18
Δ*alr* merodiploid	9.7×10^{-4}	1/40
Δ*agcS* merodiploid	4.5×10^{-3}	1/8

[a] Total 8-azahypoxanthine-resistant colonies/total viable counts.
[b] Average of three platings.

We found that plating on medium containing 8-azahypoxanthine provided effective selection for the second recombination event. Frequencies of 8-azahypoxanthine-resistant colonies obtained after growth and plating of each merodiploid were consistently near 10^{-3}, whereas the frequency of spontaneous resistance arising from strain S2 was only 10^{-6} (Table 3). In all cases we found the desired mutants among the 8-azahypoxanthine-resistant colonies, although the proportion of colonies containing the mutant varied. In the case of *ald* about half of the colonies screened contained the deletion, as expected since the lengths of flanking DNA cloned on each side of the deletion were equal. Lower proportions of Δ*alr* and Δ*agcS* mutants were found. The *agcS* construct contained substantially different lengths of flanking DNA on each side of the deletion, so both recombination events were favored on the same side of the mutant construct. The *alr* construct contained nearly equal lengths of flanking DNA; in this case the sequence on one side may have contained a recombination hot spot. One mutant colony for each gene was finally purified and designated Mm1002 (Δ*ald*), Mm1031 (Δ*alr*), and Mm1018 (Δ*agcS*).

Incorporation of *ald*$^+$ into the *upt* site. We wished to complement the Δ*ald* mutant (Mm1002) and also to implement a technique for incorporating constructs into the genome in single copies. We cloned *ald* with its promoter onto pBLPrt (Fig. 3), where it was flanked by sequences at the upstream and downstream ends of *upt*. Unlike the formation of Δ*hpt*, we were unsuccessful in making a replacement of *upt* in a single step by direct selection with 6-azauracil. We therefore adopted a two-step procedure that began with introduction of the plasmid DNA into Mm1002 with selection for neomycin resistance, followed by growth without neomycin as described above. We then plated cells on agar medium that contained 6-azauracil alone, 8-azahypoxanthine alone (taking advantage of *hpt*, which is on the vector), or both analogs together. 6-Azauracil alone gave poor selection, apparently because the initial recombination event into the DNA flanking *upt* had already compromised its effectiveness, and colonies were still neomycin resistant, indicating that the second recombination event had not occurred. However, selection with 8-azahypoxanthine alone or both analogs together was effective: all of the colonies tested were neomycin sensitive and contained the *ald* gene. One strain was purified and designated Mm1068 (Mm1002 Δ*upt*::*ald*). Deletion of *upt* removed sensitivity to 6-azauracil, since Mm1068 plated on 6-azauracil plates with a frequency of 1.4×10^{-1}, while Mm1002 had a frequency of only 5.4×10^{-5}.

Mutant phenotypes. We determined the abilities of Mm900 and each mutant to grow on L- and D-alanine. Deletion of *ald* or *agcS* eliminated growth on both isomers of alanine (Fig. 4), while Mm900 grew well on either isomer. Deletion of *alr* eliminated growth on D-alanine but did not affect growth on L-alanine. These results can be explained if *ald* encodes an alanine dehydrogenase that is specific for the L isomer, *alr* encodes an alanine racemase capable of converting the D isomer to the L isomer, and *agcS* encodes a permease required for the utilization of either isomer. Strain 1068 containing Δ*ald* and *ald*$^+$ in the *upt* site grew well on both L- and D-alanine (data not shown), indicating that Δ*ald* was complemented in *trans*. All strains grew well on ammonia.

Interestingly, when growth was assayed on a mixture of L- and D-alanine, the Δ*alr* strain showed an intermediate growth phenotype (Fig. 5). Growth occurred but was not as rapid as that of Mm900 or the Δ*alr* mutant strain on L-alanine alone. These results suggested that alanine racemase is also important to prevent D-alanine from interfering with the use of L-alanine. D-Alanine did not inhibit growth of the Δ*alr* mutant strain on ammonia.

Phylogenetic analysis. The ability of *M. maripaludis* to use L- and D-alanine appears to be unique among the archaea. We hypothesized that the three genes necessary for this function had been acquired by lateral gene transfer. BLAST searches for all three genes yielded significant homologs in *Bacteria* and few in *Archaea*. This observation suggested that the genes had been transferred almost uniquely into *M. maripaludis* from *Bacteria*. To determine the probable sources of the genes, we generated phylogenetic trees (Fig. 6). Parsimony trees (not shown) supported the evidence provided by distance matrix trees. For all three genes, members of the class *Firmicutes* (low-moles-percent G+C gram-positive bacteria) predominated among the closest relatives of *M. maripaludis*. Both distance and parsimony trees indicated that Ald of *Thermoanaerobacter* sp. (a member of the order *Clostridiales*) was the closest relative of *M. maripaludis* Ald (amino acid identity, 65%) and *Thermoanaerobacter* and *Clostridium* gene products were equidistant from *M. maripaludis* Alr (amino acid identities, 48 and 52%). *M. maripaludis* AgcS was in a clade that included *Bacil-*

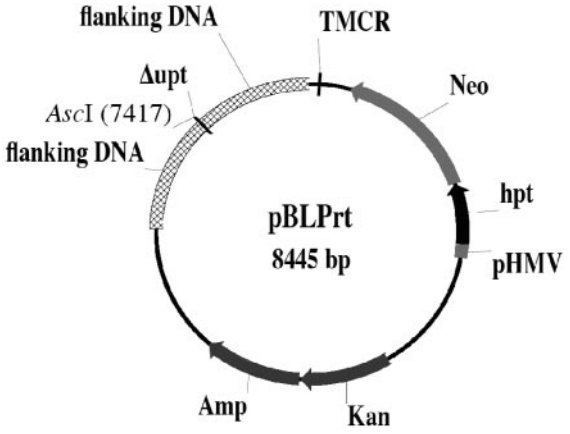

FIG. 3. Plasmid pBLPrt. DNA to be introduced into *M. maripaludis*, where it will replace the central portion of the *upt* gene, is cloned into the AscI site. Δupt and flanking DNA indicate sequences upstream and downstream of the central portion of the *upt* gene. Abbreviations are as defined in the legend to Fig. 2.

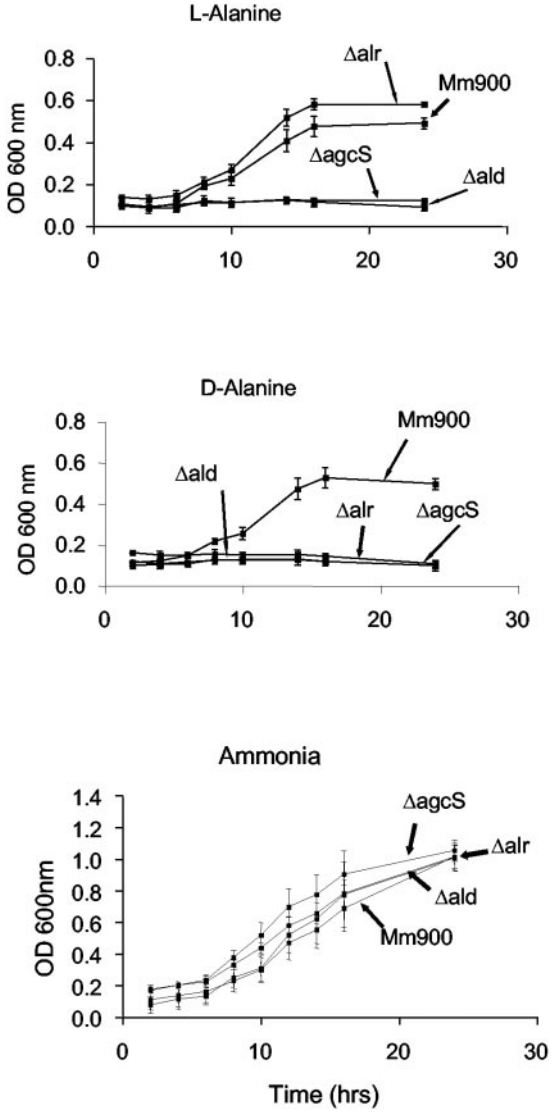

FIG. 4. Growth of Mm900 and three mutants on L-alanine, D-alanine, and ammonia. Each nitrogen source was present at 5 mM. Optical densities at 600 nm (OD_{600}) are averages of three cultures. No growth occurred in the absence of an added nitrogen source (not shown). Data for growth on L- and D-alanine and for growth on ammonia are from two separate experiments.

lus and *Oceanobacillus* species (amino acid identities, 55 and 53%). The evidence indicates that *M. maripaludis* Ald, Alr, and AgcS originated from the class *Firmicutes*. Evidence for other lateral transfer events was also seen. For example, in the case of Ald and AgcS, a proteobacterial genus (*Bilophila* or *Neisseria*) was present in the *Firmicutes-M. maripaludis* clade.

DISCUSSION

Tools for markerless mutagenesis and genome incorporation. We have reported the implementation of a system for markerless mutagenesis in *M. maripaludis*. The *hpt* gene encoding Hpt, in combination with the base analog 8-azahypoxanthine, proved to be an effective counterselectable marker. The *hpt* gene itself was successfully deleted in a single double-recombination step. The Δ*hpt* mutant strain Mm900 then provided the background for a markerless mutagenesis system in which the *hpt* gene on plasmid pCRPrtNeo provided selection for the second recombination step in a two-step procedure. Selection for 8-azahypoxanthine resistance by loss of the *hpt* gene yielded colonies at a frequency that exceeded spontaneous resistance to 8-azahypoxanthine by 2 orders of magnitude. To mediate the homologous recombination events necessary for gene replacement, we used *M. maripaludis* sequences flanking the mutant constructs that ranged in length from 428 to 1,010 bp. Recombinants were easily obtained, and it is likely that somewhat shorter segments of flanking DNA would suffice. The most important factor seemed to come into play at the final step, when colonies were screened for mutant versus wild-type alleles: as expected, equal likelihoods of recombination in the flanking DNA on each side of the mutation maximized the proportion of mutants. It should be noted that a slow-growth phenotype conferred by the mutant allele would tend to decrease the yield of mutants. This proved to be the case in the generation of a markerless mutation in the *nrpR* gene encoding the nitrogen repressor (17); however, mutant colonies were smaller and were easily detected (unpublished data).

We were also able to use the *upt* gene encoding Upt as a site for incorporation of a single-copy construct. In this study we incorporated *ald*$^+$ and found that it complemented Δ*ald*. Un-

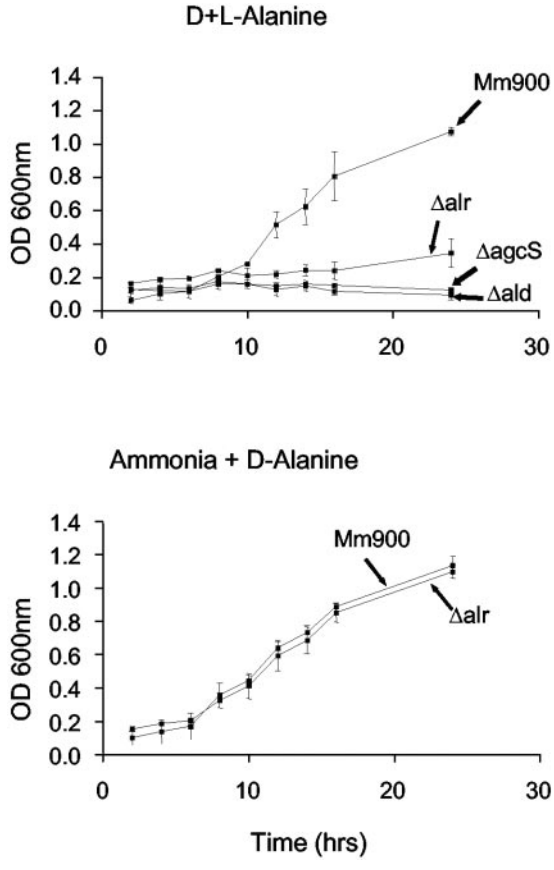

FIG. 5. D-Alanine inhibits growth on L-alanine in a Δ*alr* mutant strain. See the legend to Fig. 4 for details.

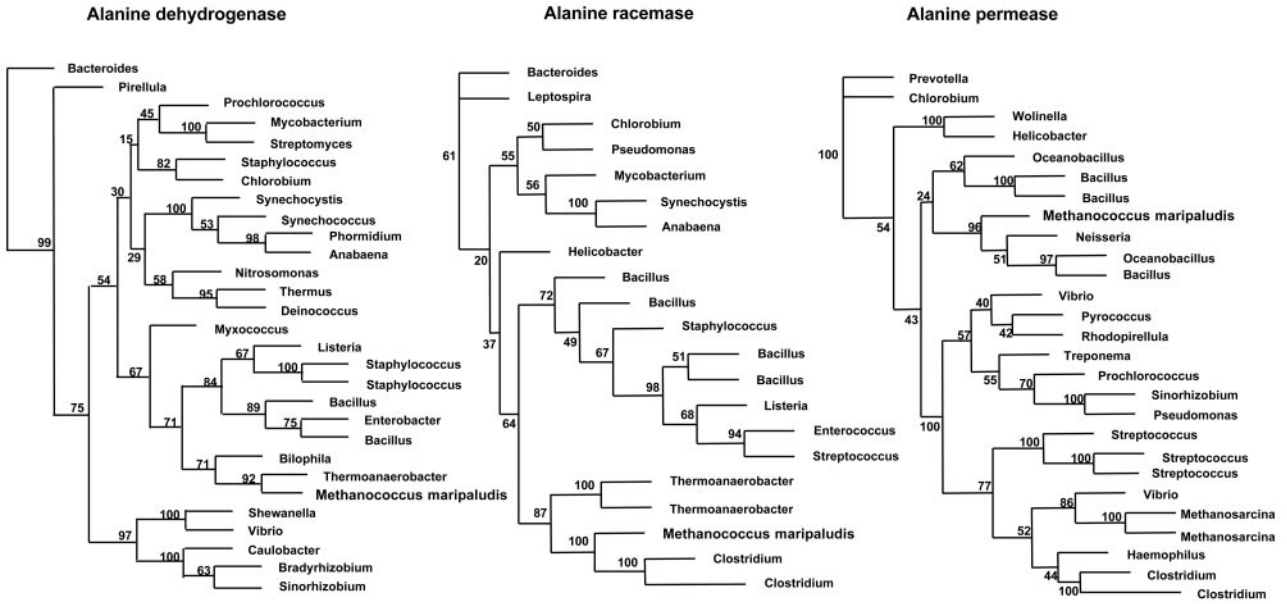

FIG. 6. Phylogenetic analysis of the Ald, Alr, and AgcS protein sequences. Consensus distance matrix trees from 100 bootstraps are shown.

like *hpt*, where a single step yielded gene replacement to produce Δ*hpt*, two steps were needed to complete the replacement of *upt* by the *ald*$^+$ construct, perhaps because 6-azauracil selection is not as effective as 8-azahypoxantine selection. We have used a similar strategy to successfully place a *nif* promoter-*lacZ* fusion in the *upt* site, in order to be able to measure gene expression from the *nif* promoter in a single copy (T. J. Lie, unpublished data). Hence, the *upt* site is generally useful for incorporation of single-copy constructs. In principle (but not in Mm900 and its derivatives, which contain Δ*hpt*), the *hpt* site could be used in the same way as a recipient of single-copy constructs, which it should be possible to incorporate in a single step, as in the creation of Mm900. Conversely, *upt* could be used to make markerless mutations as with *hpt*.

Functions of three genes in alanine utilization. Using the markerless mutagenesis system, we were able to make in-frame deletion mutations and obtain evidence for the function of three genes in the utilization of alanine by *M. maripaludis*. Ald (alanine dehydrogenase) converts L-alanine to ammonia and pyruvate. Ammonia is assimilated, and presumably pyruvate is assimilated as well (29). Alr (alanine racemase) converts D-alanine to L-alanine for use by Ald. AgcS (alanine permease) imports both L- and D-alanine. Recent work (B. Moore, unpublished data) has demonstrated that *ald* and *alr* are transcribed in a single operon, and it is worth noting that the complementation of Δ*ald* by *ald*$^+$ demonstrates the nonpolarity of the deletion mutation, since a mutation that inactivated Alr would have retained the inability to use D-alanine in the complemented strain.

In addition to its function in the utilization of D-alanine, Alr seems to play a role in protecting the system from inhibition by D-alanine, since growth of the Δ*alr* mutant on a mixture of D- and L-alanine was compromised. A possible explanation for this role of Alr would be that D-alanine might compete with L-alanine for transport. Transport of D-alanine would be productive only in the presence of the racemase. An alternative explanation would involve a close association of Alr and Ald that could have evolved to facilitate the transfer of L-alanine from Alr to Ald. In the absence of Alr, D-alanine could have access to the active site of Ald and block the metabolism of L-alanine.

Lateral gene transfer and genomic arrangements. Phylogenetic analysis indicated that all three genes were transferred to the *M. maripaludis* lineage from the class *Firmicutes*, accounting for the unusual ability of *M. maripaludis* to use L- and D-alanine as nitrogen sources. Since the three genes arose in *M. maripaludis* by lateral transfer, it is interesting to compare their functions in relatives of the donor. Although rare in archaea, all three genes are common in bacteria and appear to perform a variety of catabolic and anabolic functions. In *B. subtilis*, Ald plays a catabolic role (conversion of alanine to pyruvate and ammonia) and is needed for growth when alanine is the sole carbon or nitrogen source (23). Ald is also needed, apparently for the generation of energy from degraded proteins, during sporulation. Hence, Ald performs similar functions in different contexts in *M. maripaludis* and *B. subtilis*. Interestingly, in *Bilophila wadsworthia*, which also appears to have obtained Ald from the class *Firmicutes*, the same reaction functions in a different pathway, that of taurine degradation (15). There, Ald functions to regenerate pyruvate after a transaminase has transferred an amino group from taurine to pyruvate to form alanine.

In contrast to those of Ald, the roles of Alr are different in the class *Firmicutes* and *M. maripaludis*. In *B. subtilis*, Alr produces D-alanine for bacterial cell wall biosynthesis (8). Hence, unlike *M. maripaludis*, Ald and Alr in *B. subtilis* do not function in the same pathway. The function of AgcS in the class *Firmicutes* has not been well characterized.

A striking feature of Ald, Alr, and AgcS in *M. maripaludis* is their genetic linkage. The three genes are consecutive on the same strand. The common function of the three genes for alanine utilization in *M. maripaludis* is consistent with their

genetic linkage. In contrast, these genes are rarely linked in the class *Firmicutes*, consistent with their differing functions (only Ald and AgcS are linked in some *Bacillus* species). The linkage of the three genes in *M. maripaludis* and the apparent lack of linkage in their donor raise the question of how and when the linkage occurred. One possibility is that each gene was transferred separately and that the adaptive advantages of coregulation subsequently brought them into proximity. In this scenario, initial transfer of Ald could have conferred some ability on *M. maripaludis* to use L-alanine. Subsequent transfers of the permease and the racemase would have increased this capability and expanded it to D-alanine. Lawrence (16) has proposed theories that offer two other explanations. One possibility is that the three genes would have benefited selfishly if genetic proximity facilitated their simultaneous lateral transfer. However, in this case one would expect to have found genetic linkage and common function in the donor. Our preferred explanation is the second principle proposed by Lawrence, i.e., that genetic linkage of functionally related genes arises after lateral transfer by deletion of intervening transferred sequences that have no adaptive value. In this scenario, a single transfer of all three genes, along with sequences that separated them, could have taken place from a member of the class *Firmicutes*. Rapid loss of the intervening sequences would have ensued. In any case, the transfer of all three genes, those for alanine dehydrogenase, alanine racemase, and alanine permease, evidently created a new function in *M. maripaludis*, the ability to use L- and D-alanine as nitrogen sources.

ACKNOWLEDGMENTS

This work was supported by grants GM55255 and GM60403 from the National Institutes of Health and grant NCC 2–1273 from the NASA Astrobiology Institute.

We thank Jeremy Dodsworth for around-the-clock vigils, Erik Hendrickson for critical reading of the manuscript, and Tom Lie and Andrew Wong for assistance.

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