

## Development of a Markerless Genetic Exchange Method for *Methanosarcina acetivorans* C2A and Its Use in Construction of New Genetic Tools for Methanogenic Archaea

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**A new genetic technique for constructing mutants of *Methanosarcina acetivorans* C2A by using *hpt* as a counterselectable marker was developed. Mutants with lesions in the *hpt* gene, encoding hypoxanthine phosphoribosyltransferase, were shown to be >35-fold more resistant to the toxic base analog 8-aza-2,6-diaminopurine (8ADP) than was the wild type. Reintroduction of the *hpt* gene into a  $\Delta hpt$  host restored 8ADP sensitivity and provided the basis for a two-step strategy involving plasmid integration and excision for recombination of mutant alleles onto the *M. acetivorans* chromosome. We have designated this method markerless exchange because, although selectable markers are used during the process, they are removed in the final mutants. Thus, the method can be repeated many times in the same cell line. The method was validated by construction of  $\Delta proC \Delta hpt$  mutants, which were recovered at a frequency of 22%. Additionally, a *Methanosarcina*-*Escherichia* shuttle vector, encoding the *Escherichia coli proC* gene as a new selectable marker, was constructed for use in *proC* hosts. Finally, the markerless exchange method was used to recombine a series of *uidA* reporter gene fusions into the *M. acetivorans proC* locus. In vitro assay of  $\beta$ -glucuronidase activity in extracts of these recombinants demonstrated, for the first time, the utility of *uidA* as a reporter gene in *Methanosarcina*. A >5,000-fold range of promoter activities could be measured by using *uidA*: the methyl-coenzyme M reductase operon fusion displayed ~300-fold-higher activity than did the *serC* gene fusion, which in turn had 16-fold-higher activity than did a fusion to the unknown *orf2* gene.**

The genetic techniques available for use in *Methanosarcina* species are among the most advanced available for archaea but fall far short of those available for use with bacteria such as *Escherichia coli* and *Bacillus subtilis*. Nevertheless, methods continue to improve. Clonal populations of *Methanosarcina*, which are essential for genetic studies, can now easily be acquired via streaking on solid media; various other surface manipulations are also routine (e.g., patching, replica plating, etc.) (30). Moreover, an efficient transformation system for the introduction of DNA into *Methanosarcina* has been developed (21) and the recent developments of in vivo transposon mutagenesis (37) and directed gene replacement (38) allow efficient isolation and construction of mutants in *Methanosarcina*.

Two antibiotic resistance cassettes, the *pac* gene of *Streptomyces alboniger* for puromycin resistance (15, 21) and the *ileS12* gene from *Methanosarcina barkeri* Fusaro for pseudomononic acid resistance (4), are useful as selectable markers for genetic manipulation in *Methanosarcina*. Unfortunately, pseudomononic acid is not commercially available, which limits the use of *ileS12* and leaves the *pac* cassette as the single readily used marker. This dearth of selectable markers has limited the application of genetic analysis to certain problems in *Methanosarcina*. In particular, genome sequencing has shown that *Methanosarcina* species possess multiple copies of genes involved in the same step of numerous metabolic pathways (9, 13). In many cases, three or more duplicate genes are

present. Thus, even if both *pac* and *ileS12* are used, it is impossible to inactivate all copies of a given gene.

A potential solution to this problem lies in the use of a two-step integration-segregation strategy. In this method, a nonreplicating plasmid bearing the mutation of interest is inserted into the chromosome via homologous recombination with the wild-type allele. These merodiploid recombinants are selected by using a single selectable marker encoded on the plasmid backbone. Upon removal of selection, the unstable merodiploid allele can resolve via a second homologous recombination event between mutant and wild-type sequences. These recombinants can be either wild type or mutant, depending on the location of the recombination event, but, importantly, lose the plasmid backbone. Thus, this method allows reuse of the same antibiotic resistance marker multiple times because the marker is removed in the process of mutant construction. To improve the efficiency of this process, a counterselectable marker is often incorporated into the plasmid backbone. This allows selection, rather than screening, to be used to isolate recombinants that have resolved the merodiploid state.

Numerous counterselectable markers have been developed for use in bacteria (8, 11, 14, 28, 34) and eukaryotes (12, 18, 31, 33). Commonly used examples include the *sacB*-mediated sucrose-sensitive (14) and *tetRA*-mediated tetracycline-sensitive (5) selections used in bacteria and *ura3*-mediated 5-fluoroorotic acid (5-FOA)-sensitive selection used in various fungi. The latter counterselection differs from the others in that its use requires the utilization of *ura3* mutant host strains. Thus, introduction of *ura3*<sup>+</sup> confers 5-FOA sensitivity upon *ura3* mutants, providing the basis for subsequent selection against the introduced wild-type copy. The *ura3* counterselection has

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TABLE 1. *M. acetivorans* strains

Strain	Genotype	Reference or construction
C2A	Wild type	31
WWM1	$\Delta hpt$	Markerless exchange with pMP42
WWM24	$\Delta proC \Delta hpt$	Markerless exchange with pMP69
WWM20	<i>proC::mcrBp-uidA</i> $\Delta hpt$	Markerless exchange with pJK85
WWM23	<i>proC::serCp-uidA</i> $\Delta hpt$	Markerless exchange with pJK86
WWM25	<i>proC::orf2p-uidA</i> $\Delta hpt$	Markerless exchange with pJK87
WWM26	<i>proC5(BglII frameshift)</i> $\Delta hpt$	Markerless exchange with pJK84

also proven useful in archaea, where it was successfully used in *Halobacterium salinarum* during the construction of *bop* deletion mutants (27). The 5-FOA-sensitive selection has also been used in conjunction with *pyrE2* mutants in the haloarchaeon *Haloflex volcanii*, where it was used as a counterselectable marker to delete the *cmi* gene (3).

Genes involved in purine and pyrimidine salvage pathways, in particular those encoding various phosphoribosyl transferases (PRTases), have also been used as counterselectable markers (3, 10, 12, 18, 27, 31, 33). These enzymes provide the cell alternative means for de novo nucleotide biosynthesis, by incorporating exogenous bases into their nucleotide pools, and for recycling nonstandard bases. Thus, purine PRTases are responsible for converting purines (i.e., adenine, guanine, and the nonstandard xanthine and hypoxanthine) into the corresponding nucleotide monophosphates. Purine analogs (e.g., 8-aza-2,6-diaminopurine [8ADP], 8-aza-guanine, 8-aza-hypoxanthine, etc.) are also substrates for these PRTases; however, incorporation of these compounds can be lethal to the cells. Accordingly, PRTase-defective mutants are resistant to toxic base analogs and, in such hosts, reintroduction of the wild-type copy provides the basis for a counterselection strategy. For example, the *B. subtilis* uracil PRTase gene *upp* has been used as a counterselectable marker with 5-fluorouracil to create a variety of mutants in *B. subtilis*  $\Delta upp$  hosts (10).

The possibility of developing such a system in archaea is supported by several studies of purine and pyrimidine salvage pathways (6, 7, 17, 32, 36). In addition to demonstrating the presence of base salvage pathways, it was observed that spontaneous resistance to purine analogs is associated with decreased activity of certain purine salvage enzymes. In a previous study (37), we isolated an *M. acetivorans* mutant with a transposon insertion into a gene for a putative hypoxanthine PRTase, which we hoped could be used for development of a counterselection strategy. In this paper, we describe the development of this system for use in *M. acetivorans* and its use in construction of additional genetic tools, including new selectable markers and reporter gene fusions.

#### MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** Standard conditions were used for growth of *E. coli* strains (35). DH5 $\alpha$ /λ-*pir* (24) was used as the host for maintenance of all *pir*-dependent plasmids, while DH10B (Stratagene, La Jolla, Calif.) was used for all other replicons. *M. acetivorans* strains, described in Table 1, were grown in single-cell morphology (30) at 35°C in high-salt (HS) broth medium containing either 125 mM methanol-40 mM acetate (HS-MA), 125 mM methanol (HS-M), 50 mM trimethylamine (HS-T), 75 mM dimethylamine (HS-D), or 80 mM acetate (HS-A). Growth of *M. acetivorans* on media solidified with 1.5% agar was as described earlier (4). All plating manipulations were carried out under strictly anaerobic conditions in an anaerobic glove box. Solid medium

plates were incubated in an intrachamber anaerobic incubator as described earlier (23). For selection of *Methanosarcina* strains carrying the puromycin transacetylase gene (*pac*), puromycin (Pur; CalBiochem, San Diego, Calif.) was added from sterile, anaerobic stocks at a final concentration of 2 μg/ml. *Methanosarcina* extract prepared as described below was added at a final concentration of 0.05% (vol/vol) for supplementation of potential auxotrophic mutants incapable of synthesizing methanogen-specific factors. The purine analog 8ADP (Sigma, St. Louis, Mo.) was added from sterile, anaerobic stocks at a final concentration of 20 μg/ml for selection against the hypoxanthine PRTase (*hpt*) gene. The purine analogs 2-aminopurine, 2-methylpurine (2MP), 2-mercaptapurine, 6-mercaptapurine, 6-methyl-aminopurine, 2,6-diaminopurine, and 2-amino-6-methylmercaptapurine (Sigma) were added from sterile, anaerobic stocks at concentrations ranging from 462 to 0.302 μg/ml. Proline (Pro; Sigma) was added from sterile, anaerobic stocks at a final concentration of 50 mM for construction and maintenance of all *M. acetivorans pro* strains (38).

**Methanosarcina extract preparation.** *M. acetivorans* cells from a 1-liter stationary-phase culture grown in HS-MA medium were collected by centrifugation (6,000 × *g* for 15 min). Cells were lysed by resuspension in ca. 100-ml 0.1 M NaHCO<sub>3</sub>, pH 6.8, containing 0.1 mg of DNase I/ml (spontaneous lysis occurs due to osmotic shock). The lysate was clarified by centrifugation (13,000 × *g* for 1 h), and high-molecular-weight components were removed by ultrafiltration with a 3,000-molecular-weight cutoff membrane (YM3; Amicon, Beverly, Mass.). Cell-free filtrate was filter sterilized by using a 0.25-μm-pore-size filter and was stored anaerobically at 4°C in the dark. All manipulations were conducted under strictly anaerobic conditions in an anaerobic chamber with an atmosphere of N<sub>2</sub>/CO<sub>2</sub>/H<sub>2</sub> (75:20:5).

**DNA methods.** Standard methods were used throughout for isolation and manipulation of plasmid DNA from *E. coli* (2). Plasmid DNA was isolated from *M. acetivorans* as described earlier (21), while genomic DNA was isolated with a modified cetyltrimethylammonium bromide method (22). DNA hybridizations were performed by using the DIG System (Roche, Mannheim, Germany) according to manufacturer guidelines, with the following modifications. Hybridizations were performed at 65°C in a high-sodium dodecyl sulfate (SDS) buffer (25 mM Na<sub>2</sub>HPO<sub>4</sub>, 7% SDS, pH 7.2). Posthybridization washes were performed at 65°C for 30 min with gentle agitation twice each in 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 5% SDS, pH 7.2, and 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 1% SDS, pH 7.2. MagnaGraph Nylon transfer membranes were from Osmonics (Westborough, Mass.). DNA sequences were determined from double-stranded templates by using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif.) following manufacturers' guidelines at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois.

**Transformation.** *E. coli* strains were transformed by electroporation by using an *E. coli* Gene Pulser (Bio-Rad) as recommended. Liposome-mediated transformation was used for *Methanosarcina* species as described earlier (21), modified in reference 4.

**Plasmid constructions.** Plasmid constructions and oligonucleotide primers used are presented in Table 2. The physical map of selected plasmids is presented in Fig. 1. pJK41 and all derivatives are *pir* dependent in *E. coli* and are nonreplicating in *Methanosarcina* (20).

**Determination of generation time.** Cells from mid-log-phase *M. acetivorans* cultures (optical density at 600 nm [OD<sub>600</sub>] ~ 0.5) were harvested by centrifugation (5,000 × *g* for 10 min), washed once, and then resuspended in an equal volume of plain HS broth (without a carbon and energy source). Washed cells (0.3 ml) were inoculated into 10-ml broth cultures in triplicate and were incubated at 37°C on a tube roller. Growth was monitored at OD<sub>600</sub> by using a Milton Roy Company Spectronic 21.

**Determination of β-glucuronidase activity.** 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc; Sigma) was used to qualitatively determine the presence of the *uidA* gene product (β-glucuronidase) in *M. acetivorans*. *M. acetivorans* colo-

TABLE 2. Plasmids and primers used in this study

Plasmid or primer	Description and/or construction or primer sequence	Reference or source or added site
<b>Plasmids</b>		
pJK41	Amp <sup>R</sup> , Pur <sup>R</sup> cloning vector, <i>oriR6K</i> replicon	19
pWM241	pC2A replicon cloned into pBluescript SK	20
pWM368	<i>M. barkeri</i> Fusaro <i>mcrB</i> promoter source	39
pWM369	<i>M. barkeri</i> Fusaro <i>serC</i> promoter source	39
pWM370	<i>M. barkeri</i> Fusaro <i>orf2</i> promoter source	39
pCK12	<i>M. acetivorans</i> C2A <i>proC</i> plasmid	40
pMP29	<i>NdeI/KpnI</i> -cut hpt PCR product (using primers <i>NdeI</i> -apt and <i>ApaI</i> KpnIap) cloned into <i>NdeI/KpnI</i> -cut pWM368	This study
pMP33	<i>AflII/XhoI</i> -cut up-hpt PCR fragment (using primers <i>AflII</i> aphpt and <i>SacI</i> -aphpt) cloned into <i>AflII/XhoI</i> -cut pJK41	This study
pMP42	<i>BglII/BamHI</i> -cut dn-hpt PCR fragment (using primers <i>BglII</i> dnapt and <i>BamHI</i> dnapt) cloned into <i>BglII/BamHI</i> -cut pMP33	This study
pMP44	762-bp <i>SphI/ApaI</i> fragment of pMP29 cloned in <i>SphI/ApaI</i> -cut pJK41	This study
pMP45	<i>NdeI/BglII</i> -cut <i>uidA</i> PCR product (using primers <i>uidA</i> ( <i>NdeI</i> ) and <i>uidA</i> ( <i>BglII</i> )) cloned into <i>NdeI/BglII</i> -cut pWM368	This study
pJK81	<i>NdeI/BglII uidA</i> fragment from pMP45 cloned into <i>NdeI-BamHI</i> -cut pWM368	This study
pJK82	<i>NdeI/BglII uidA</i> fragment from pMP45 cloned into <i>NdeI-BamHI</i> -cut pWM369	This study
pJK83	<i>NdeI/BglII uidA</i> fragment from pMP45 cloned into <i>NdeI-BamHI</i> -cut pWM370	This study
pMP62	<i>BamHI</i> -cut/T4 DNA polymerase-treated <i>proC</i> fragment from pCK12 cloned into <i>AflII</i> -cut/T4 DNA polymerase-treated pMP44	This study
pMP69	<i>SpeI/MluI</i> -cut up- <i>proC</i> PCR product (using primers <i>SpeI</i> - <i>proB</i> and <i>MluI</i> - <i>proB</i> ) and <i>MluI/KpnI</i> -cut dn- <i>proC</i> PCR product (using primers <i>MluI</i> - <i>proC</i> and <i>KpnI</i> - <i>proC</i> ) cloned into <i>SpeI/KpnI</i> -cut pMP44	This study
pJK84	<i>BglII</i> -cut pMP62 treated with T4 DNA pol and dNTPs then religated	This study
pJK85	<i>SphI-Ecl136II mcrBp-uidA</i> fragment of pJK81 cloned into <i>BglII</i> -cut/T4 DNA Pol-treated/ <i>SphI</i> -cut pMP62	This study
pJK86	<i>SphI-Ecl136II serCp-uidA</i> fragment of pJK82 cloned into <i>BglII</i> -cut/T4 DNA Pol-treated/ <i>SphI</i> -cut pMP62	This study
pJK87	<i>SphI-Ecl136II orf2p-uidA</i> fragment of pJK83 cloned into <i>BglII</i> -cut/T4 DNA Pol-treated/ <i>SphI</i> -cut pMP62	This study
pJK88	<i>NdeI/BamHI</i> -cut <i>E. coli proC</i> PCR fragment (using primers <i>procF</i> and <i>procR</i> ) cloned into <i>NdeI/BamHI</i> -cut pWM369	This study
pJK89	<i>SpeI</i> fragment of pWM241 with pC2A replicon cloned into <i>XbaI</i> -cut pJK88	This study
<b>Primers</b>		
<i>NdeI</i> -apt	CCGCCGCATATGCTTGAAGACTGAAAGACTACATGG	<i>NdeI</i>
<i>ApaI</i> KpnIap	CCGCCGGTACCGGGCCCTCACTGATCCCCAAAGACATCC	<i>KpnI, ApaI</i>
<i>AflII</i> upapt	CCGCCGCTTAAGCCACTCCCGGAACAGAGG	<i>AflII</i>
<i>SacI</i> -upapt	CCGCCGCTCGAGTTTGGCGGTTTCAATATTCC	<i>XhoI</i>
<i>BglII</i> dnapt	GGGGGGAGATCTCATATGTCTTTGGGGATCAGTGAAGC	<i>BglII</i>
<i>BamHI</i> dnapt	GGGGGGGGATCCACCAGAGACTTTGCAATTACC	<i>BamHI</i>
<i>uidA</i> ( <i>NdeI</i> )	GGGGGGCATATGTTACGTCTGTAGAAAGG	<i>NdeI</i>
<i>uidA</i> ( <i>BglII</i> )	GGGGGGAGATCTGATCATTAACGGCGCAGTACCG	<i>BglII</i>
<i>SpeI</i> - <i>proB</i>	GCCGCCACTAGTTTAGTTCGGGAGCAATAGGC	<i>SpeI</i>
<i>MluI</i> - <i>proB</i>	GCCGCCACGGCTTTAGTTTATCCCTTCCAGTAT	<i>MluI</i>
<i>MluI</i> - <i>proC</i>	GCCGCCACGGCTCGATTCTTTTAC	<i>MluI</i>
<i>KpnI</i> - <i>proC</i>	GCCGCCGGTACCATTGTTCCGCCTGAAA	<i>KpnI</i>
<i>procF</i>	GCGCGCCATATGGAAAAGAAAATCGGTTTTATTGG	<i>NdeI</i>
<i>procR</i>	GCGCGCGGATCCGACGTAACCGCACCGAAGTG	<i>BamHI</i>

nies were blotted onto Whatman filter paper, flooded with 2 mg of X-Gluc/ml in 150 mM Tris-HCl, pH 8.0, and incubated aerobically at room temperature for 5 to 30 min. Clones expressing *uidA* turned blue ( $\beta$ -Gluc<sup>+</sup>). *p*-Nitro-phenol-glucuronide (pNP-Gluc) was used in quantitative assays of  $\beta$ -glucuronidase expressed from translational fusions of *uidA* to various *M. barkeri* genes. Cell extracts were prepared aerobically by pelleting mid-log-phase cultures (OD<sub>600</sub> ~ 0.5) by centrifugation (5,000 × g for 10 min), osmotic lysis of the cell pellet by resuspension in ice-cold 50 mM Tris-HCl, pH 8.0, and removal of cell debris by centrifugation at 4°C (14,000 × g for 15 min). Extracts were held on ice until assayed. Assays (1 ml) were performed at room temperature in 50 mM Tris-HCl, pH 8.0, with 4 mM pNP-Gluc. Assays were started by addition of pNP-Gluc and were continuously monitored at 415 nm by using a Hewlett-Packard 8453 UV-Visible Spectrophotometer. The specific activity (micromoles · minute<sup>-1</sup> · milligram of protein) was calculated by using the molar extinction coefficient for pNP-Gluc ( $\epsilon = 12,402$  liters · mol<sup>-1</sup> · cm<sup>-1</sup>), which was experimentally deter-

mined in the buffer conditions used in this assay. Protein concentration was determined by the Bradford assay kit from Pierce (Rockford, Ill.).

## RESULTS

**Isolation and characterization of *hpt1::mini-mar* mutant.** In a previous study we identified a transposon-induced mutant of *M. acetivorans* C2A as an auxotroph requiring supplementation with a cell-free *Methanosarcina* extract (37). However, the initial auxotrophic phenotype of this mutant was apparently an artifact of our growth conditions because the mutant grew without supplementation after a prolonged adaptation period.



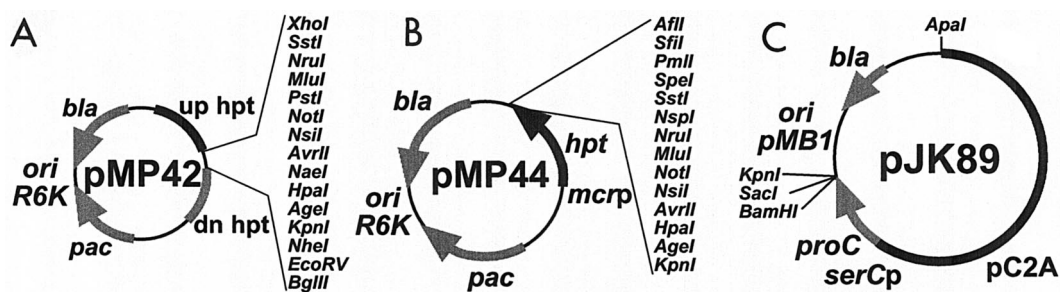


FIG. 1. Plasmids used in this study. (A) pMP42 is an *M. acetivorans* C2A *hpt* deletion plasmid. The regions upstream (up *hpt*; dark grey box) and downstream (dn *hpt*; light grey box) of *hpt* were PCR amplified and were cloned into pJK41 (thin black line [20]), creating the  $\Delta hpt$  allele. pJK41 and its derivatives are *pir* dependent in *E. coli* (oriR6K) and are nonreplicating in *Methanosarcina* species. *pac* and *bla* (light grey arrows) encode puromycin resistance in *Methanosarcina* species and ampicillin resistance in *E. coli*. Multiple cloning sites are available for integration of genes into the *hpt* locus. (B) pMP44 is the base plasmid for markerless exchange in *M. acetivorans* and is also derived from pJK41. The *M. acetivorans hpt* (medium grey arrow) is expressed from the *M. barkeri* Fusaro methyl-coenzyme M reductase promoter (*mcrBp*; light grey box). Multiple cloning sites are available for insertion of mutated alleles to be recombined onto the *M. acetivorans* chromosome. (C) pJK89 is a *Methanosarcina-E. coli* shuttle vector that carries selectable markers for *proC* complementation and ampicillin resistance. The *E. coli proC* gene (*proC*; dark grey arrow) is expressed from the *M. barkeri* Fusaro phosphoserine aminotransferase promoter (*serCp*; light grey box) and is functional in both *E. coli* and *M. acetivorans*. The pC2A replicon (21) allows autonomous replication in *Methanosarcina* species. pJK89 replicates via the high-copy-number pMB1 replicon in *E. coli*.

The transposon insertion site of this mutant was cloned and sequenced, revealing an insertion into an open reading frame with strong homology (53% identical with Fasta) (26) to the product of the *Methanothermobacter thermotrophicus hpt* gene, namely, hypoxanthine PRTase (29). The transposon-induced mutant was therefore designated *hpt1::mini-mar*.

The *M. acetivorans hpt* gene (designated MA0717 in the *M. acetivorans* genome sequence; GenBank accession no. NC 003552) is 570 bp in length and encodes a protein of 189 amino acids. The downstream gene, MA0718, overlaps 20 bp at the 3' end of *hpt* and, thus, may comprise an operon with *hpt*. MA0718 is a 1,008-bp gene encoding a 335-amino-acid protein with significant homology to diphthamide synthase and C-5 cytosine-specific DNA methylase.

Hypoxanthine PRTases convert hypoxanthine to inosine-5'-monophosphate and are involved in the purine salvage pathways of a number of organisms (16, 32, 36). This is consistent with our observation that the *hpt1::mini-mar* mutant originally behaved as an auxotroph. The medium used in the isolation of this strain contains exogenous nucleotide bases. We suspect that de novo synthesis is repressed in this medium and that the adaptation period required for prototrophic growth involves switching from the salvage pathway back to the de novo pathway. In some methanoarchaea, purine salvage mutants have been isolated by selecting resistance to purine analogs (6, 7, 17, 32, 36). Therefore, we tested wild-type *M. acetivorans* and the *hpt1::mini-mar* strain for sensitivity to a number of purine analogs (Table 3). For wild-type *M. acetivorans*, the most toxic purine analogs tested were 8ADP and 2MP, both of which inhibited growth at ca. 2  $\mu\text{g}/\text{ml}$ . The *hpt1::mini-mar* mutant was significantly more resistant to both compounds, consistent with the proposed role of the *hpt* gene in purine salvage. The MIC of 8ADP for the *hpt1::mini-mar* mutant is >74.5  $\mu\text{g}/\text{ml}$ , at least 35-fold higher than that for the wild type. 2MP, however, retained some toxicity for the mutant, causing very poor growth at 10  $\mu\text{g}/\text{ml}$  and complete growth inhibition at 40  $\mu\text{g}/\text{ml}$ . Therefore, the toxicity of 2MP is independent of *hpt* at this high concentration, suggesting that the cell possesses an-

other means to activate 2MP. Spontaneous 8ADP<sup>r</sup> mutants of *M. acetivorans* arise at a frequency of  $2.9 \times 10^{-5}$  (determined by plating dilutions of *M. acetivorans* C2A onto medium with and without 20  $\mu\text{g}$  of 8ADP/ml).

**Construction of a  $\Delta hpt$  host strain for use in 8ADP counterselection strategies.** The *hpt* gene has been used as a counterselectable marker in other organisms (12); thus, the fortuitous isolation of this *hpt1::mini-mar* mutant afforded an opportunity to expand the genetic techniques available for use in *M. acetivorans*. To make use of this finding, an unmarked *hpt* mutant was constructed to serve as the host for the "markerless" exchange system described below.

The *hpt* gene was deleted from the *M. acetivorans* C2A chromosome by using the two-step procedure shown in Fig. 2A. Approximately 800 bp of DNA upstream and downstream of *hpt* was cloned, creating the  $\Delta hpt$  allele carried on plasmid pMP42, which also carries the *pac* cassette encoding resistance to puromycin (Fig. 1A). (This deletion leaves the *hpt* promoter and downstream gene intact; however, we have made no attempt to verify expression of this gene.) Due to the plasmid's inability to replicate in *Methanosarcina*, selection for Pur<sup>r</sup>

TABLE 3. MIC of purine analogs on *M. acetivorans* C2A and *hpt1::mini-mar*

Purine analog	MIC ( $\mu\text{g}/\text{ml}$ ) <sup>a</sup>	
	C2A	<i>hpt1::mini-MAR</i>
2-MP	1.31	10
8ADP	1.74	>74.5
6-Mercaptopurine	5.67	NT <sup>b</sup>
2,6-Diaminopurine	24.6	NT
6-Methylaminopurine	107	NT
2-Aminopurine	>462	NT
2-Mercaptopurine	>462	NT
2-Amino-6-methylmercaptopurine	>462	NT

<sup>a</sup> ca.  $10^7$  cells of the indicated strain were inoculated into HS-MA medium containing serial dilutions of a purine analog. Cultures were incubated at 37°C, and growth was scored after 14 days.

<sup>b</sup> NT, not tested.

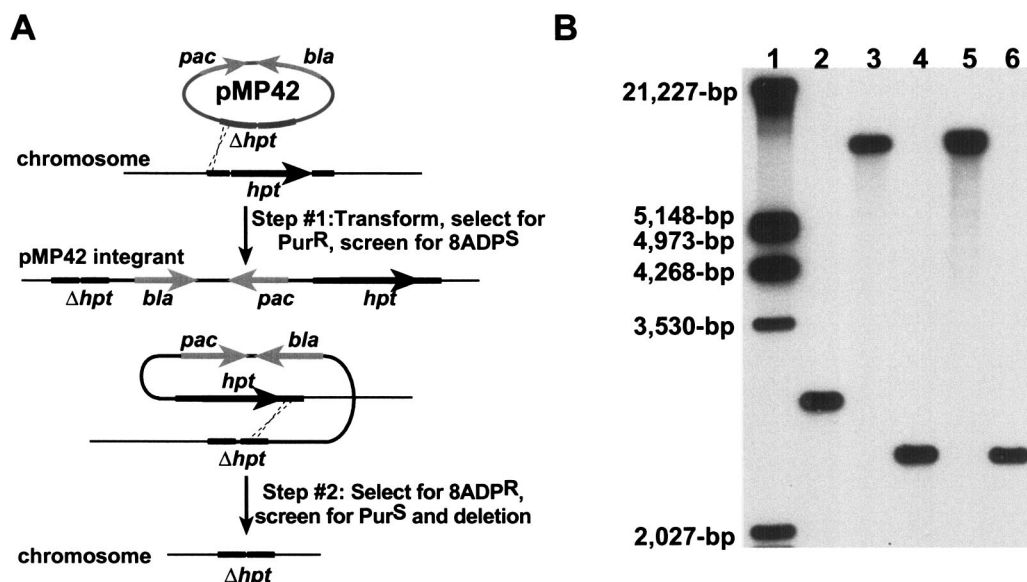


FIG. 2. Construction of  $\Delta hpt$  host for markerless exchange. (A) The *M. acetivorans* *hpt* gene (black arrow) was deleted in a two-step process with pMP42. *M. acetivorans* was transformed to puromycin resistance ( $Pur^R$ ) with pMP42 as described elsewhere. Because pMP42 is incapable of replication in *M. acetivorans*  $Pur^R$  transformants can only arise via homologous recombination (dotted lines) into the region flanking *hpt* (step no. 1). Integration through the upstream region is shown; however, integration into the region downstream of *hpt* is also possible. Integration of pMP42 into the chromosome creates an unstable merodiploid, which can segregate via a second homologous recombination event under nonselective conditions, resulting in plasmid excision from the chromosome (step no. 2). Some of these resolved merodiploids leave the  $\Delta hpt$  allele on the chromosome, and these recombinants are resistant to 8ADP ( $8ADP^R$ ). (B) The genome structure of two pMP42 integrants and their  $8ADP^R$  recombinants was verified by DNA hybridization of *Alu*NI-digested genomic DNA to DIG-labeled pMP42 as described elsewhere. Lane 1, DIG-labeled *Eco*RI/*Hind*III lambda DNA (Roche); lane 2, *M. acetivorans* C2A; lane 3, pMP42 upstream integrant; lane 4,  $8ADP^R$  recombinant of the upstream integrant; lane 5, pMP42 downstream integrant; and lane 6,  $8ADP^R$  recombinant of the downstream integrant. The predicted sizes of the hybridizing bands for *hpt*,  $\Delta hpt$ , and both pMP42 integrants are 2,807, 2,437, and 7,371 bp, respectively, based on the known DNA sequence of the region.

pMP42 transformants results in *hpt*/ $\Delta hpt$  merodiploid strains with the plasmid integrated into the chromosome via homologous recombination (Fig. 2A, step no. 1). These merodiploids remain sensitive to 8ADP due to the presence of an intact copy of the *hpt* gene. Growth under nonselective conditions (i.e., without puromycin or 8ADP) allows resolution of the merodiploid by homologous recombination between the repeated sequences upstream and downstream of *hpt*. Approximately half of these recombinants leave the  $\Delta hpt$  allele on the chromosome, and these can easily be selected from the mixed population by selection for  $8ADP^R$  (Fig. 2A, step no. 2).

To validate this strategy, we characterized 32  $Pur^R$  pMP42 transformants of *M. acetivorans*. As expected, all 32 were  $Pur^R$  and  $8ADP^S$ . Ten of these pMP42 integrants were analyzed by DNA hybridization to determine the site of plasmid integration. Five integrants gave the predicted hybridization pattern for simple insertions into the chromosome, while the remaining five appeared to have multiple plasmids integrated into the chromosome in tandem (data not shown). Two simple integrants, one with pMP42 integrated into the region upstream of *hpt*, the other with pMP42 integrated through the downstream region, were grown nonselectively for 4 generations and were then plated onto medium with 8ADP to select recombinants that had resolved the merodiploid state in favor of the  $\Delta hpt$  allele (Fig. 2A, step no. 2). Eight  $8ADP^R$  recombinant clones (one from the upstream insertion and seven from downstream insertion) were characterized in detail. Two of these were both

$Pur^R$  and  $8ADP^R$ , suggesting that they had acquired point mutations in the intact *hpt* gene, and were not analyzed further. The remaining six recombinants were  $Pur^S$  and  $8ADP^R$ , indicating that they had resolved the merodiploid state by homologous recombination, resulting in the loss of the plasmid backbone.

These recombinants, along with their parental pMP42 integrants and wild-type *M. acetivorans*, were analyzed by DNA hybridization. As shown in Fig. 2B, *M. acetivorans* C2A (lane 2) shows the wild-type *hpt* hybridizing band. Both integrants (lanes 3 and 5) show a single hybridizing band with an increased size corresponding to insertion of pMP42 into the chromosome. The  $8ADP^R$  recombinants from each integrant (lanes 4 and 6) show a single, smaller hybridizing band consistent with the loss of the *hpt* gene and excision of the plasmid DNA. Similar results confirming the integration of pMP42 and subsequent deletion of *hpt* were obtained in hybridization experiments involving digests with three different restriction endonucleases (data not shown). One verified  $\Delta hpt$  mutant, designated WWM1, was used for all subsequent studies.

The generation times of *M. acetivorans* C2A and WWM1 were determined on methanol, trimethylamine, and dimethylamine (Table 4). The growth rate differed by less than 10% for *M. acetivorans* C2A and WWM1 for all substrates tested, indicating that this mutation does not grossly affect the physiology of *M. acetivorans* under laboratory conditions.

TABLE 4. Generation time of *M. acetivorans* C2A and  $\Delta hpt$  strains<sup>a</sup>

Substrate	Generation time (h) for:	
	C2A	$\Delta hpt^b$
Methanol	7.1 ± 0.2	7.8 ± 0.1
Trimethylamine	9.8 ± 0.4	10.4 ± 0.7
Dimethylamine	24.8 ± 1.2	27.4 ± 0.8

<sup>a</sup> Determined as described in Materials and Methods.

<sup>b</sup> WWM1 was used as the  $\Delta hpt$  strain.

**Construction of  $\Delta proC$   $\Delta hpt$  by using “markerless” genetic exchange.** The  $\Delta hpt$  strain WWM1 allows efficient recombination of unmarked mutations onto the chromosome via a method that we designated markerless exchange because, although it uses selectable markers, it does not leave them on the chromosome at the end of the process (Fig. 3A). The method uses plasmid pMP44, which carries the selectable marker for Pur<sup>r</sup>, *pac*, and the counterselectable marker *hpt* (Fig. 1B).

To perform markerless exchange, the desired mutation is first cloned into pMP44. The resulting plasmid is then used to transform WWM1 to Pur<sup>r</sup>. Because this plasmid is incapable of replication in *Methanosarcina*, these transformants arise by integration into the host chromosome via recombination between the mutant allele on pMP44 and the chromosomal wild-type allele. (Integration cannot occur at the *hpt* locus because the  $\Delta hpt$  mutation removes all homology to the *hpt* gene in

pMP44.) The resulting merodiploids are 8ADP<sup>s</sup> due to the presence of *hpt* on pMP44; thus, recombinants that have resolved the merodiploid state (and therefore lost the integrated pMP44 backbone) can be selected as 8ADP<sup>r</sup> clones. Such recombinants are easily differentiated from spontaneous mutations within *hpt* because they also become Pur<sup>s</sup>. Depending on where the resolving recombination event occurs, these Pur<sup>s</sup>/8ADP<sup>r</sup> recombinants will retain either the wild-type or mutant allele. Assuming that these recombination events occur at equal frequency and that the mutant does not have a substantial growth disadvantage, the frequencies of the mutant and wild type should be roughly equal. Therefore, screening a relatively small number of recombinants should give the desired mutant.

To test this strategy, we constructed a mutant with an unmarked deletion of *proC*. This gene was previously shown to be dispensable in *M. acetivorans* and provides an easily testable phenotype when mutated (i.e., proline auxotrophy) (38). To construct the *proC* deletion, ca. 1 kb of PCR amplified DNA upstream and downstream of *proC* was cloned into pMP44, resulting in the  $\Delta proC$  allele carried on plasmid pMP69 (Fig. 3A). *M. acetivorans* WWM1 ( $\Delta hpt$ ) was grown with proline supplementation and was transformed to Pur<sup>r</sup> with circular pMP69 (Fig. 3A, step no. 1). Analysis of 18 Pur<sup>r</sup> transformants by DNA hybridization showed that seven clones had pMP69 integrated upstream of *proC*, while four clones had pMP69 integrated downstream of *proC*. The remaining seven Pur<sup>r</sup>

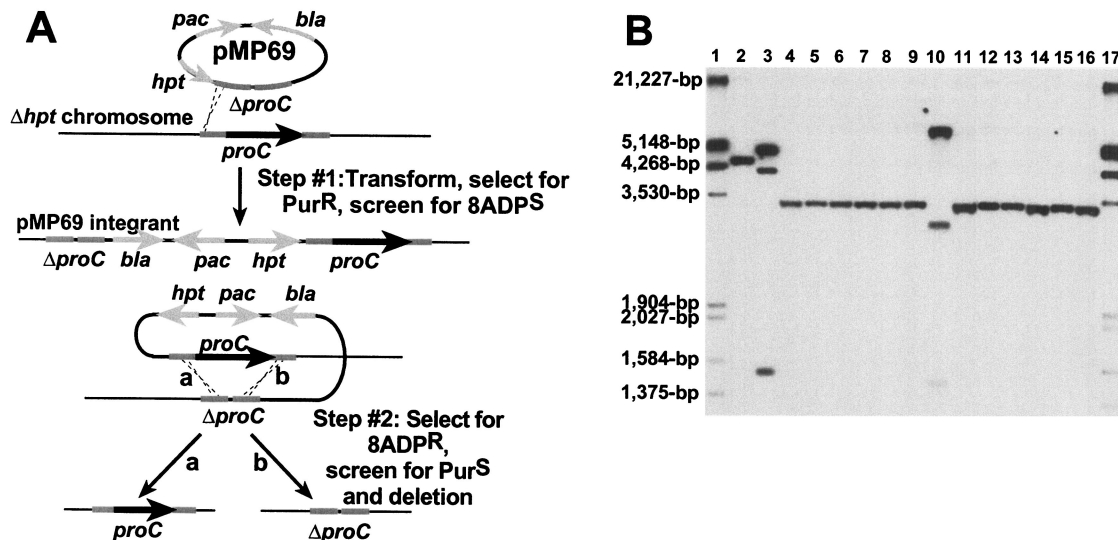


FIG. 3. (A) Deletion of *proC* using markerless exchange. Markerless exchange utilizes the *hpt* gene as a counterselectable marker to construct gene deletions on the *M. acetivorans* chromosome. The *proC* gene was deleted from *M. acetivorans*  $\Delta hpt$  by using pMP69 with the  $\Delta proC$  allele shown in light grey. *M. acetivorans*  $\Delta hpt$  was transformed to puromycin resistance (Pur<sup>r</sup>) with pMP69 as described. Because pMP69 is incapable of replication in *M. acetivorans*, Pur<sup>r</sup> transformants can only arise via homologous recombination (dotted lines) into the region flanking *proC* (step no. 1). (Insertion into the upstream region is shown. Integration is also possible via the downstream homology.) Integration of the plasmid creates an unstable merodiploid that is Pur<sup>r</sup> and sensitive to the toxic purine analog ADP (8ADP<sup>s</sup>). Nonselective growth allows plasmid excision and resolution of the merodiploid allele via a second recombination event (dotted lines), resulting in either the wild type or the deletion allele, depending on which homologous region is used for the second event. (B) Verification of *proC* deletions. Genomic DNA from two pMP69 merodiploids and 12 8ADP<sup>r</sup>, Pro<sup>-</sup> recombinants was examined by hybridization of *Hind*III-digested genomic DNA to DIG-labeled pMP69 as described earlier. Lane 1, DIG-labeled *Eco*RI/*Hind*III lambda DNA (Roche); lane 2, WWM1; lane 3, pMP69 upstream integrant; lanes 4 to 9, 8ADP<sup>r</sup>, Pro<sup>-</sup> recombinants of the upstream integrant; lane 10, pMP69 downstream integrant; and lanes 11 to 16, 8ADP<sup>r</sup>, Pro<sup>-</sup> recombinants of the downstream integrant. Based on the known DNA sequence of the region, the hybridizing bands for *proC* and  $\Delta proC$  are expected to be 4,463 and 3,388 bp, respectively. The pMP69 upstream integrant is expected to produce hybridizing bands of 4,818, 4,142, and 1,505 bp, while pMP69 downstream integrant is expected to give bands of 5,898, 3,062, and 1,505 bp.



integrants gave an aberrant banding pattern that could not easily be explained by simple integration events and were not analyzed further (data not shown). One integrant of each type was grown nonselectively for 8 generations and was then subjected to 8ADP selection (Fig. 3A, step no. 2). To examine the efficiency of this method, we examined 100 8ADP<sup>r</sup> recombinants from the upstream integrant and 106 recombinants from the downstream integrant. Of the 100 8ADP<sup>r</sup> clones obtained from the upstream insertion strain, three were Pur<sup>r</sup>, suggesting that they carried point mutations in the plasmid-encoded *hpt* gene, and 32 were proline auxotrophs. Similarly, two 8ADP<sup>r</sup> clones from the downstream insertion strain appeared to have point mutations in *hpt*, while 13 out of the remaining 104 exhibited proline auxotrophy. Twelve 8ADP<sup>r</sup> Pro<sup>-</sup> recombinants (six derived from each integrant) were analyzed by DNA hybridization to confirm loss of *proC* (Fig. 3B). All twelve recombinants gave a smaller hybridizing band of exactly the predicted size. One correct  $\Delta proC$  recombinant, designated WWM24, was used for further studies.

The generation times of WWM1 and WWM24 were determined with and without proline supplementation. The generation times of WWM1 are  $7.3 \pm 0.3$  and  $7.2 \pm 0.4$  h with and without proline supplementation, respectively. The generation time of WWM24 with proline supplementation is  $9.7 \pm 0.8$  h. Without proline, WWM24 did not grow. As will be discussed below, the difference in generation time between WWM1 and WWM24 with proline supplementation probably accounts for the low percentage of  $\Delta proC$  recombinants obtained.

**Integration of reporter gene fusions into *proC*.** In addition to its use for construction of defined deletions by using the markerless exchange method, the *hpt* counterselection can also be used to insert foreign genes into the *M. acetivorans* chromosome. To demonstrate this method, we constructed a series of translational fusions of various *M. barkeri* Fusaro promoters to the commonly used *E. coli* reporter gene *uidA*, which encodes  $\beta$ -glucuronidase, and recombined them into *proC* on the *M. acetivorans* WWM1 chromosome.

The promoter regions for the *M. barkeri* methyl-coenzyme M reductase (*mcrBp*) operon, the *M. barkeri* phosphoserine aminotransferase (*serCp*) gene, and the *M. barkeri* unknown open reading frame (*orf2p*) (37) were fused to *uidA*, resulting in plasmids pJK85, pJK86, and pJK87, respectively. These three promoters were expected to exhibit a wide range of activities based on previous studies (1, 37). pJK84 contains a frameshift in *proC* and was used to construct an isogenic strain lacking  $\beta$ -glucuronidase for use as a control. The *mcrBp-uidA*, *serCp-uidA*, and *orf2p-uidA* reporter gene fusions were integrated into the chromosome of WWM1 at the *proC* locus by using the markerless exchange. In this case, recombinants were screened for proline auxotrophy, puromycin sensitivity, and the presence of  $\beta$ -glucuronidase. Two independently isolated Pro<sup>-</sup>, Pur<sup>s</sup>,  $\beta$ -Gluc<sup>+</sup> clones for each fusion were identified, and  $\beta$ -glucuronidase activity was determined for each strain. As expected the fusions showed widely divergent  $\beta$ -glucuronidase levels, with the activity levels of the *proC::mcrBp-uidA*, *proC::serCp-uidA*, and *proC::orf2p-uidA* fusions being  $6.7 \pm 0.7$  U,  $2.1 \times 10^{-2} \pm 2.4 \times 10^{-2}$  U, and  $1.3 \times 10^{-2} \pm 0.5 \times 10^{-3}$  U, respectively.

**Use of *E. coli proC* as a selectable marker in *M. acetivorans*  $\Delta proC$  strains.** The proline auxotrophy of the  $\Delta proC$  strains

was exploited to create an additional selectable marker for use in *M. acetivorans*. It was previously shown that *proC* mutations in *M. acetivorans* could be complemented by plasmid-encoded *proC* genes (38), thus allowing prototrophy to be used as a selection in these strains. To avoid unwanted recombination between the selectable *pro*<sup>+</sup> marker and host chromosome, we constructed a gene cassette in which the *E. coli proC* gene was driven from the *serC* promoter of *M. barkeri* (22). This hybrid *proC* gene was then used as the selectable marker in the shuttle plasmid pJK89, which replicates via the high-copy-number pMB1 origin in *E. coli* and the medium-copy-number pC2A replicon in *M. acetivorans* (Fig. 1C).

Transformation of *M. acetivorans* WWM24 ( $\Delta proC \Delta hpt$ ) to prototrophy with pJK89 resulted in  $4.4 \times 10^7$  total transformants per 2  $\mu$ g of DNA, comparable to other shuttle vectors containing the *pac* cassette that routinely give ca.  $10^7$  transformants per 2  $\mu$ g of DNA (21). Plasmid DNA isolated from *Methanosarcina* transformants could be used to retransform *E. coli*, and restriction endonuclease digestion patterns of plasmids obtained after passage through *M. acetivorans* were unchanged, indicating that the plasmid is structurally stable in *M. acetivorans* (data not shown).

## DISCUSSION

The markerless exchange method using *hpt* as a counterselectable marker significantly expands the genetic capabilities available for study of *M. acetivorans*. Similar genetic tools have been reported for only a few archaea. A counterselection strategy has been described for use in two haloarchaea (3, 27); however, the method reported here is the first to be described for use in methanoarchaea. The *hpt*-based method was used here to construct insertions and deletions at the *proC* locus but subsequently has proven to be generally useful. In studies to be reported elsewhere, the method has been used to construct numerous deletions of genes involved in the utilization of methanol as a growth substrate (M. A. Pritchett and W. W. Metcalf, unpublished). A key feature of the method is that it can be used repeatedly to create strains with multiple mutations. To date we have constructed strains with as many as five unmarked deletions. Moreover, in cases where multiple genes are cotranscribed in an operon, the method allows construction of nonpolar mutations of individual genes within the transcriptional unit.

Two potential concerns, one more significant than the other, should be considered when employing this method. As mentioned above, resolution of the merodiploid state should result in ca. 50% wild-type and 50% mutant recombinants. This is based on the assumption that recombination between the duplicated upstream and downstream regions occurs at equal frequency and that the mutant and wild-type strains grow at the same rate following segregation. Although the first assumption has not been tested, it is very likely to be true, so long as the upstream and downstream regions of homology are of similar size. In contrast, the second assumption is expected to be true only for a subset of mutations. In this study, only 22% (45 of 201) of recombinants selected from *proC*<sup>+</sup>/ $\Delta proC$  merodiploids were Pro<sup>-</sup>. Comparison of the generation times of *proC*<sup>+</sup> and  $\Delta proC$  mutant strains showed a significant difference ( $7.2 \pm 0.4$  h versus  $9.7 \pm 0.8$  h, respectively). Thus, the

wild-type cells would have outgrown the *proC* mutants during the nonselective growth and, as a result, would have constituted a larger percentage of the population at the time of plating onto selective medium. More pronounced differences in generation time would result in even larger skews in population sizes. Thus, use of this method for isolation of mutants with profound growth defects is likely to be problematic and the inability to isolate a mutation with this method should not be construed as proof that the gene in question is essential.

A more minor concern stems from the relatively high frequency of spontaneous 8ADP resistance ( $2.9 \times 10^{-5}$ ). Accordingly, selection for resolution of merodiploids by 8ADP<sup>r</sup> can result in clones that have not lost the plasmid backbone but rather have acquired mutations in the *hpt* gene. These clones are easily distinguished from merodiploid-resolving recombinants because they retain the *pac* gene in the plasmid backbone and, hence, are Pur<sup>r</sup>. In this study, only five of the 206 8ADP<sup>r</sup> clones selected from the *proC*<sup>+</sup>/ $\Delta$ *proC* merodiploid were Pur<sup>r</sup>, suggesting that spontaneous *hpt* mutations are rare compared to loss of plasmid by homologous recombination. Thus, spontaneous 8ADP resistance is not a significant problem for markerless exchange; however, the high spontaneous resistance rate does prevent direct selection of insertions and deletions at the *hpt* locus. In previous studies, we attempted to disrupt the *hpt* locus by direct selection for 8ADP resistance after transformation with linear DNA carrying a mutated *hpt* allele, but these attempts always failed to produce the desired recombinants. In all cases, the clones obtained appeared to be spontaneous 8ADP<sup>r</sup> mutants (data not shown). For this situation, it is likely that the combined losses in efficiency due to both transformation and a double recombination event (as opposed to the single event used in the strategy presented above) lowered the frequency of the desired events to well below that of spontaneous 8ADP<sup>r</sup>. These difficulties led us to develop the two-step integration-segregation strategy.

Despite the ability of markerless exchange to allow construction of multiple mutations in a single strain, it is often easier, and faster, to construct mutations with simple insertions of selectable markers. For this reason, we constructed a selectable marker based on the *E. coli proC* gene that confers prototrophy on  $\Delta$ *proC* hosts. The lack of homology between the *E. coli* and *M. acetivorans proC* genes and its relative smallness are particularly useful features of this marker. The *proC* plasmids developed here can be used in strains containing mutations marked with the *pac* cassette, thus allowing facile complementation analysis. Although not explicitly tested, it is probable that the *proC* cassette of pJK89 will also function in single copy. Thus, this marker may also prove useful in transposon mutagenesis or gene replacement. However, pJK89 can only be used in *proC* strains like WWM24, which restricts its use somewhat. To our knowledge, this is the first instance of using a bacterial gene to complement an archaeal mutant.

Finally, the demonstration of reporter genes in *Methanosarcina* is an important advance. Although reporter gene fusions have been used in a wide variety of organisms, including methanoarchaea like *Methanococcus* species, such fusions have not yet been well described for use in *Methanosarcina* (although unpublished data cited in reference 19 suggest that *lacZ* can be used). In this study,  $\beta$ -glucuronidase activity was easily quantified over a 5,000-fold range in extracts of strains with *uidA*

fusions to three different *M. barkeri* Fusaro promoters. Activity was undetectable in *M. acetivorans* strains lacking *uidA* fusions (data not shown). Further,  $\beta$ -glucuronidase activity could be qualitatively visualized by exposure of cells harboring the *uidA* gene to an X-Gluc solution. Unfortunately, visualization of  $\beta$ -glucuronidase activity with X-Gluc cannot be performed anaerobically because pigment formation does not occur under the strongly reducing conditions used to cultivate methanogens (25). Therefore, clones had to be replica plated or patched for aerobic testing with X-Gluc, while the original plates were maintained under anaerobic conditions to preserve cell viability.

The promoters used here were carefully chosen and probably represent nearly the full span of promoter activity in *Methanosarcina*: *mcrBp* drives expression of the genes for the enzyme methylreductase (1), which comprises ca. 5% of total cell protein, whereas transcripts from *orf2p* could barely be detected with a sensitive primer extension assay (21). Each fusion was constructed so that the ATG start codon of *uidA* was superimposed upon the start codon of the gene in question. Thus, the  $\beta$ -glucuronidase activity of each fusion represents contributions from both the transcriptional and translational control signals for each gene. The  $\beta$ -glucuronidase activity from the *mcrB* promoter was  $\sim$ 300-fold higher than that from the *serC* promoter, while the *serC* promoter activity was  $\sim$ 16-fold higher than that of the *orf2* promoter. These promoter fusions provide a valuable baseline for comparison of future reporter gene fusions. Moreover, the availability of promoters of known strengths allows controlling expression of foreign genes by swapping promoters. Thus, we chose to express the *E. coli proC* gene in the selectable marker described above from the moderately expressed *serC* promoter (i.e., it seemed appropriate to drive expression of an amino acid biosynthetic gene from a known amino acid biosynthetic gene promoter). In contrast, expression of the *mariner* transposase gene from the *mcrB* promoter was much more effective than from *serCp* and *orf2p* in promoting transposition of the mini-MAR transposon in *M. acetivorans* (37).

The methods presented here, in combination with those previously published, comprise a basic toolkit that will now allow greatly expanded genetic studies of *Methanosarcina* species (4, 21, 22, 37, 38). Coupled with the metabolic diversity of members of this genus and the availability of complete, or nearly complete, genome sequences for three *Methanosarcina* species (9, 13), the prospects for future study of these complex and interesting microorganisms are very exciting.

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