

## New methods for tightly regulated gene expression and highly efficient chromosomal integration of cloned genes for *Methanosarcina* species

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**Summary** A highly efficient method for chromosomal integration of cloned DNA into *Methanosarcina* spp. was developed utilizing the site-specific recombination system from the *Streptomyces* phage  $\phi$ C31. Host strains expressing the  $\phi$ C31 integrase gene and carrying an appropriate recombination site can be transformed with non-replicating plasmids carrying the complementary recombination site at efficiencies similar to those obtained with self-replicating vectors. We have also constructed a series of hybrid promoters that combine the highly expressed *M. barkeri* *PmcrB* promoter with binding sites for the tetracycline-responsive, bacterial TetR protein. These promoters are tightly regulated by the presence or absence of tetracycline in strains that express the *tetR* gene. The hybrid promoters can be used in genetic experiments to test gene essentiality by placing a gene of interest under their control. Thus, growth of strains with *tetR*-regulated essential genes becomes tetracycline-dependent. A series of plasmid vectors that utilize the site-specific recombination system for construction of reporter gene fusions and for tetracycline regulated expression of cloned genes are reported. These vectors were used to test the efficiency of translation at a variety of start codons. Fusions using an ATG start site were the most active, whereas those using GTG and TTG were approximately one half or one fourth as active, respectively. The CTG fusion was 95% less active than the ATG fusion.

**Keywords:** genetics, site-specific recombination, *tetR*, essential gene.

### Introduction

Methanoarchaea are a unique group of organisms that are responsible for the vast majority of biologically mediated methane production. Methanogenesis plays a critical role in the carbon cycle, global warming, alternative energy strategies, waste

treatment and agriculture, but the experimental study of methanoarchaea is laborious. They are oxygen-sensitive anaerobes and, until recently, methods for their genetic manipulation were scarce. However, this has begun to change, in particular for members of the genus *Methanosarcina* (reviewed in Sowers and Schreier (1999) and Rother and Metcalf (2005)). Although, these developments have substantially improved the genetic malleability of *Methanosarcina*, the pace of genetic studies is frustratingly slow and certain types of experiments remain difficult, in particular those requiring stable insertion of cloned DNA into the chromosome and those requiring stringent regulation of gene expression.

Cloned DNA can be introduced into *Methanosarcina* spp. with autonomously replicating plasmid vectors (Metcalf et al. 1997); however, this approach often introduces experimental artifacts owing to the higher plasmid copy number. For example, we have found that transformation can be difficult, or impossible, with plasmids carrying genes encoding membrane proteins or highly expressed reporter gene fusions. Further, plasmids can be unstable, especially when they encode genes that confer a growth disadvantage (Apolinario et al. 2005). Insertion of the cloned DNA into the chromosome can avoid these problems; however, current methods of cloned DNA insertion for use with *Methanosarcina* are less efficient by a factor of about 100 than transformation with autonomous plasmids because of their dependence on homologous recombination. In other organisms, methods utilizing site-specific recombination, instead of homologous recombination, have allowed much higher integration efficiencies (e.g., Lyznik et al. 2003, Schweizer 2003, and references therein). One particularly useful site-specific recombinase system utilizes the *Streptomyces* bacteriophage  $\phi$ C31 integrase (Thorpe and Smith 1998).

The  $\phi$ C31 integrase catalyzes recombination without aid of other proteins (Thorpe and Smith 1998), a feature that has al-

lowed its use in diverse hosts including *Streptomyces* (Bierman et al. 1992), *Escherichia coli* (Thorpe and Smith 1998), *Schizosaccharomyces pombe* (Thomason et al. 2001) and *Homo sapiens* cell lines (Groth et al. 2000). The site-specific  $\phi$ C31 integration reaction is unidirectional. Many site-specific recombinases, such as the *Saccharomyces* FLP system (Schweizer 2003, Branda and Dymecki 2004), can be used efficiently to excise a DNA fragment flanked by recombination sites (Schweizer 2003); however, integration is less efficient because the recombinase is fully reversible. Accordingly, the recombinase-encoding gene cannot be constitutively expressed in the recipient because it destabilizes the construct. Use of reversible recombinases, therefore, requires transient expression, whereas a unidirectional recombinase can be expressed constitutively without compromising the stability of the insert, which greatly simplifies strain constructions (Belteki et al. 2003).

Regulated expression of cloned genes in *Methanosarcina* is problematic because few regulated promoters have been well characterized in members of this genus. In contrast, large numbers of well-characterized and tightly regulated promoters are known in bacteria. These have allowed the development of numerous systems for stringent regulation of cloned genes and for the testing of gene essentiality (Baron and Bujard 2000, Guzman et al. 1995, Lutz and Bujard 1997, Kamionka et al. 2005). Among the most useful of these is the tetracycline-regulated promoter system from the transposon Tn10 (Beck et al. 1982). The Tn10-encoded TetR protein binds specifically to the tetO operator sequence in the absence of tetracycline, thus preventing transcription. However, binding of tetracycline by the TetR protein abrogates binding of the protein to the promoter allowing transcription. This relatively simple system has been combined with a variety of natural and synthetic promoters to create numerous different tetracycline-regulated systems (reviewed in Berens and Hillen (2004) and Sprengel and Hasan (2007)). These include both prokaryotic and eukaryotic systems, ones that act as either Tet-responsive repressors or activators, and ones in which the binding of mutant derivatives of TetR depends on the presence of tetracycline, instead of its absence.

The use of  $\phi$ C31-mediated site-specific recombination and Tet-regulated gene expression has revolutionized genetic analysis, especially in organisms, such as higher eukaryotes, where genetic manipulation has traditionally been both difficult and slow. Given the inherent difficulties of genetic experiments in methanoarchaea, we believed that the development of similar approaches for *Methanosarcina* species would be especially worthwhile. These efforts are reported below.

## Materials and methods

### *Strains, media and growth conditions*

*Methanosarcina* strains used in the study are described in Table 1. These were grown in single cell morphology (Sowers et al. 1993) at 37 °C in high salt (HS) liquid medium (Metcalf et al. 1996) containing 125 mM methanol, 50 mM trimethyl-

amine (TMA) or 40 mM acetate as indicated. Growth on medium solidified with 1.5% agar was as described by Zhang et al. (2000). All plating manipulations were carried out in an anaerobic glove box (Coy Laboratory Products, Grass Lake, MI). Solid media plates were incubated in an intra-chamber anaerobic incubator as described by Metcalf et al. (1998). Puromycin (CalBiochem, San Diego, CA) was added from sterile, anaerobic stocks at a final concentration of 2  $\mu$ g ml<sup>-1</sup> for selection of *Methanosarcina* strains carrying the puromycin transacetylase gene (*pac*). The purine analog 8-aza-2,6-diaminopurine (8-ADP) (Sigma, St. Louis, MO) was added from sterile, anaerobic stocks at a final concentration of 20  $\mu$ g ml<sup>-1</sup> for selection against the hypoxanthine phosphoribosyl transferase gene (*hpt*).

*Escherichia coli* cells were grown under standard conditions (Wanner 1986). *Escherichia coli* WM3118 (F, *mcrA*,  $\Delta$ (*mrr-hsdRMS-mcrBC*),  $\phi$ 80*lacZ* $\Delta$ M15,  $\Delta$ *lacX74*, *recA1*, *endA1*, *araD139*,  $\Delta$ (*ara*, *leu*)7697, *galU*, *galK*, *rpsL*, *nupG*,  $\lambda$ attB::pAMG27(*PrhaB-trfA33*)) was constructed by integration of pAMG27 (Table 2) into the  $\lambda$ attB site of DH10B (Invitrogen, Carlsbad, CA) by site-specific recombination as described by Haldimann and Wanner (2001). WM3118 was used as the host strain for all plasmids containing *oriV*, allowing plasmid copy number to be dramatically increased by growth in a medium containing 10 mM rhamnose before plasmid purification (Wild et al. 2002). BW25141 was the host strain for  $\Pi$ -dependent plasmids (Haldimann and Wanner 2001). DH10B was the host strain for all other plasmids (Invitrogen, Carlsbad, CA).

### *Transformation methods*

*Escherichia coli* strains were transformed by electroporation using an *E. coli* Gene Pulser (Bio-Rad, Hercules, CA) as recommended. Liposome-mediated transformation was used for *Methanosarcina* as described by Boccazzi et al. (2000).

### *Plasmids and DNA primers*

Plasmids used in the study are described in Table 2. All plasmids were verified by extensive restriction endonuclease digestion analysis and DNA sequencing of selected junction regions (data not shown). Because of the large number of plasmid intermediates constructed during the course of this work, only the final versions used in the study are presented in Table 2. Annotated GenBank-style DNA sequence files for each plasmid are provided in the online supplementary materials. Details of the plasmid constructions are available on request. Standard techniques were used for the isolation and manipulation of plasmid DNA using *E. coli* hosts (Ausubel et al. 1992).

### *Molecular genetic methods*

*Methanosarcina* strain constructions via markerless exchange or gene replacement following transformation with linear DNA were according to Zhang et al. (2002) and Pritchett et al. (2004) and were performed in media containing either metha-

Table 1. *Methanosarcina* strains used in this study.

Strain	Genotype	Source/Reference
<i>M. acetivorans</i> C2A	Wild type	DSM2834 <sup>1</sup>
WWM1	$\Delta hpt$	(Pritchett et al. 2004)
WWM19	$\Delta hpt::pWM357$	(Guss et al. 2005)
WWM60	$\Delta hpt::PmcrB-tetR$	This study
WWM73	$\Delta hpt::PmcrB-tetR-\phi C31-int-attP$	This study
WWM75	$\Delta hpt::PmcrB-tetR-\phi C31-int-attB$	This study
WWM82	$\Delta hpt::PmcrB-\phi C31-int-attP$	This study
WWM83	$\Delta hpt::PmcrB-\phi C31-int-attB$	This study
<i>M. barkeri</i> Fusaro	Wild type	DSM804 <sup>1</sup>
WWM85	$\Delta hpt::PmcrB-\phi C31-int-attP$	This study
WWM86	$\Delta hpt::PmcrB-\phi C31-int-attB$	This study
WWM155	$\Delta hpt::PmcrB-tetR-\phi C31-int-attP$	This study
WWM154	$\Delta hpt::PmcrB-tetR-\phi C31-int-attB$	This study
WWM235	$\Delta hpt::PmcrB-tetR-\phi C31-int-attB, PmcrB(tetO1)::mcrBCDGA$	This study

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nol or TMA as growth substrate. Transformation efficiency was tested in medium containing TMA as growth substrate. Approximately 2  $\mu$ g of purified DNA was used in each transformation. Retrofitting of plasmids carrying  $\lambda$ attB sites with plasmid pAMG40 was performed using BP clonase (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. After the in vitro site-specific recombination reaction was complete, the mixture was used to transform WM3118 with selection for chloramphenicol and kanamycin resistance. Co-integration of the plasmids was verified by restriction endonuclease digestion of purified plasmid DNAs.

#### PCR verification of plasmid integration

Single copy integration of non-replicating plasmids via  $\phi$ C31 site-specific recombination was verified using a four-primer PCR screen. Template DNA was obtained by resuspending cells from a colony grown on agar-solidified medium in sterile H<sub>2</sub>O, which causes immediate cell lysis. After a 4 min pre-incubation at 94 °C, 35 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 90 s were performed, followed by a final incubation at 72 °C for 2 min. *Methanosarcina acetivorans* integrants were screened with C31 screen-all#1 (GAAGCTTCCCC-TTGACCAAT, primer #1 in Figure 1), C31 screen-C2A#1 (TTGATTTCGGATACCCTGAGC, primer #2 in Figure 1), C31 screen-pJK200#1 (GCAAAGAAAAGCCAGTATGGA, primer #3 in Figure 1), and C31 screen-pJK200#2 (TTTTTCGTCTCAGCCAATCC, primer #4 in Figure 1). *Methanosarcina barkeri* integrants were screened with C31 screen-all#1 (primer #1 in Figure 1), C31 screen-Fus#1 (CGAACTGTGGTGCAAAGAC, primer #2 in Figure 1), C31 screen-pJK200#1 (primer #3 in Figure 1), and C31 screen-pJK200#2 (primer #4 in Figure 1). The PCRs were performed using Taq polymerase in Failsafe buffer J (Epicentre, Madison WI). For most of the plasmids described here the expected bands are: parental strain control, 910 bp; plasmid control, 450 bp; single plasmid integrations, 670 and 730 bp; inte-

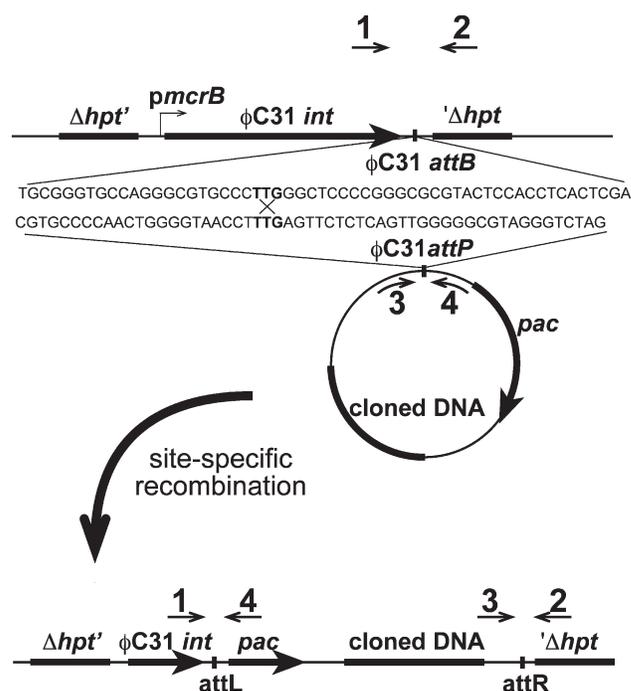


Figure 1. Scheme of the  $\phi$ C31 integrase-mediated site-specific recombination in *Methanosarcina*. Strains carrying the  $\phi$ C31 integrase gene ( $\phi$ C31 *int*) driven by a strong constitutive promoter (*PmcrB*) and the phage integration site ( $\phi$ C31 *attB*) inserted into the *hpt* locus of both *Methanosarcina acetivorans* and *M. barkeri* were constructed as described (Table 1). Transformation of these strains to Pur<sup>R</sup> (conferred by the *pac* gene) with non-replicating plasmids carrying the complementary integration site ( $\phi$ C31 *attP*) results in highly efficient integration of the plasmid into the host chromosome after site-specific recombination between *attB* and *attP* (denoted by X) catalyzed by the *Int* protein.  $\Delta hpt'$  and  $\Delta hpt$  represent the chromosomal regions flanking the *hpt* locus, which was deleted upon insertion of the *int* gene and *att* sites. *attL* and *attR* represent the hybrid recombination sites formed by site specific recombination between *attB* and *attP*. The numbered arrows indicate the location of PCR primers used to verify the single-copy insertion of plasmids as described in the methods. Sequences for the screening primers are provided in Table 2.

gration of plasmid multimers, 670, 730 and 450 bp. For pAB79-derived plasmids the expected bands are: parental strain control, 910 bp; plasmid control, 510 bp; single plasmid integrations, 679 and 740 bp; integration of plasmid multimers, 680, 741 and 511 bp.

#### *Extract preparation and $\beta$ -glucuronidase assay*

The preparation of cell extracts and the  $\beta$ -glucuronidase assay method were as previously described (Rother et al. 2005). Enzymatic activity was determined by following production of *p*-nitrophenol at 415 nm ( $\epsilon = 12402 \text{ mM}^{-1} \text{ cm}^{-1}$ ). Absorbance spectra were recorded with a Hewlett Packard 8453 diode array spectrophotometer. Activity is reported in milliunits (mU;  $1 \text{ nmol min}^{-1}$ ). Strains were adapted to each growth substrate for at least 15 generations before measurement. Reported values are means of at least three separate cultures. Protein concentration was determined by the method of Bradford (Bradford 1976), with bovine serum albumin as the standard. The limit of detection for  $\beta$ -glucuronidase is  $0.4 \text{ mU mg protein}^{-1}$ .

## Results

### *Construction of strains and plasmids for site-specific integration of cloned DNA into the Methanosarcina chromosome*

A strategy for highly efficient insertion of cloned DNA fragments into the *Methanosarcina* chromosome utilizing the well-characterized *Streptomyces*  $\phi$ C31 phage integrase system is shown in Figure 1. In this system, non-replicating plasmids carrying either the attB or attP recombination sites are used to transform strains carrying the complementary recombination site and a constitutively expressed  $\phi$ C31 integrase (*int*) gene. Site-specific recombination between the attB and attP sequences results in highly efficient integration of the plasmid into the host chromosome.

To achieve this goal, we constructed a series of *M. barkeri* and *M. acetivorans* strains that carry either attB or attP and the  $\phi$ C31 integrase gene expressed from the constitutive *PmcrB* promoter of *M. barkeri* (Rother et al. 2005) (Table 1). A series of complementary plasmids was also constructed (Table 2, Figure 2). Several of these plasmids are derivatives of the fosmid cloning vector pWM357 (Zhang et al. 2002) and are useful for constructing genomic DNA libraries; however, they have been modified to include additional useful features. The parental plasmid was modified to include a marker for selection of puromycin resistance in *Methanosarcina* species and the origin of replication from plasmid RP4 (*oriV*) to allow induction of high-copy replication in appropriate host strains (Wild et al. 2002). The plasmids also carry the phage  $\lambda$  attB site, which can be used to retrofit the plasmids with additional features (see below).

### *Efficiency of plasmid integration via the $\phi$ C31 integrase system*

We tested the efficiency of the  $\phi$ C31 integrase system in a series of transformation experiments (Figure 3). The self-repli-

cating vector pWM321 yielded approximately  $10^6$  puromycin-resistant ( $\text{Pur}^R$ ) transformants in each of the strains examined. Non-replicating attB and attP fosmids gave nearly as many transformants as pWM321, but only when the transformation involved the complementary attP and attB hosts (i.e., attB plasmids transformed into attP strains and vice versa). When fosmids were introduced into strains carrying identical att sites (i.e. attB x attB and attP x attP), less than ten transformants arose. Fosmids lacking a  $\phi$ C31 att site were incapable of transforming either  $\phi$ C31-*int* strain. These data suggest that  $\phi$ C31 site-specific recombination can occur in *Methanosarcina* at efficiencies that approach transformation by autonomous vectors. To compare the efficiency of site-specific recombination with the efficiency of homologous recombination, we transformed a control strain carrying an 8 kb region of homology to the fosmid backbone inserted into the chromosomal *hpt* locus. In this strain, non-replicating fosmids produced approximately 30-fold lower transformation efficiencies regardless of the presence or absence of  $\phi$ C31 att sites. No recombinants were obtained in wild-type strains after transformation with any of the non-replicating vectors.

### *Integration vectors for facile construction of uidA reporter gene fusions*

We have found the  $\phi$ C31 integration system to be particularly useful in gene regulation studies using reporter gene fusions, where stably maintained, single-copy fusions are desirable. To facilitate such studies, we constructed a series of  $\phi$ C31 integration plasmids to allow construction of transcriptional and translational fusions to *uidA* gene from *E. coli*, which encodes  $\beta$ -glucuronidase (GUS), a useful reporter system in *Methanosarcina* (Pritchett et al. 2004) (Figure 2).

We used these constructs to examine the effects of alternatives start codons on translational efficiency in *Methanosarcina*. Plasmids with the highly expressed *mcrB* promoter (*PmcrB*) fused to *uidA* using ATG, GTG, TTG, CTG, and AAA as translation initiation codons were constructed and integrated into the *M. acetivorans* chromosome in single copy. Using methanol as a growth substrate,  $\beta$ -glucuronidase activity was similar when the start codon was ATG or GTG ( $2034 \pm 348 \text{ mU mg}^{-1}$  and  $1593 \pm 495 \text{ mU mg}^{-1}$ , respectively), whereas changing the start site to TTG reduced activity by two-thirds ( $559 \pm 200 \text{ mU mg}^{-1}$ ). When CTG was the start site, activity was reduced by a factor of about 20 compared to ATG ( $79 \pm 19 \text{ mU mg}^{-1}$ ). Mutation of the start site to AAA resulted in complete elimination of  $\beta$ -glucuronidase activity ( $< 0.4 \text{ mU mg}^{-1}$ ).

### *Construction of tetracycline-regulated promoters for use in Methanosarcina*

To develop a Tet-regulated gene expression system for *Methanosarcina*, we constructed a series of plasmids in which *PmcrB* was modified to include binding sites for the Tn10-derived TetR protein (tetO) (Figure 4). Four promoters with variable placement of the tetO operator were constructed, designated *PmcrB*(tetO1), *PmcrB*(tetO2), *PmcrB*(tetO3) and

Table 2. Plasmids and primers used in the study.

Plasmid	Features/Use	Source
pAMG27	$\lambda$ attP CRIM plasmid encoding kanamycin resistance and PrhaB-trfA33	This study
pAMG33	Fosmid vector encoding chloramphenicol and puromycin resistance with oriV and lattP	This study
pAMG40	<i>E. coli</i> -Methanosarcina shuttle vector for fosmid retrofitting encoding ampicillin resistance and lattB	This study
pAMG44	Fosmid vector encoding chloramphenicol and puromycin resistance with oriV, lattP and fC31-attP	This study
pAMG45	Fosmid vector encoding chloramphenicol and puromycin resistance with oriV, lattP and fC31-attB	This study
pAMG63	Plasmid for markerless insertion of PmcRB-fC31-int-attP into the <i>M. acetivorans</i> hpt locus (used to construct WWM82)	This study
pAMG64	Plasmid for markerless insertion of PmcRB-fC31-int-attB into the <i>M. acetivorans</i> hpt locus (used to construct WWM83)	This study
pAMG70	Plasmid for markerless insertion of PmcRB-fC31-int-attB into the <i>M. barkeri</i> hpt locus (used to construct WWM85)	This study
pAMG71	Plasmid for markerless insertion of PmcRB-fC31-int-attP into the <i>M. barkeri</i> hpt locus (used to construct WWM86)	This study
pAMG82	$\phi$ C31-attB vector for construction of translational fusions to the <i>E. coli</i> uidA gene using an ATG start codon	This study
pAMG83	$\phi$ C31-attB vector for construction of translational fusions to the <i>E. coli</i> uidA gene using an GTG start codon	This study
pAMG95	$\phi$ C31-attB vector for construction of translational fusions to the <i>E. coli</i> uidA gene using an TTG start codon	This study
pAMG96	$\phi$ C31-attB vector with <i>M. barkeri</i> mcrB promoter fusion to uidA with a GTG start site	This study
pAMG103	$\phi$ C31-attB vector for construction of translational fusions to the <i>E. coli</i> uidA gene using an CTG start codon	This study
pAMG104	$\phi$ C31-attB vector with <i>M. barkeri</i> mcrB promoter fusion to uidA with a TTG start site	This study
pAMG105	$\phi$ C31-attB vector with <i>M. barkeri</i> mcrB promoter fusion to uidA with a CTG start site	This study
pAMG108	$\phi$ C31-attB vector for construction of translational fusions to the <i>E. coli</i> uidA gene using an AAA start codon	This study
pAMG109	$\phi$ C31-attB vector with <i>M. barkeri</i> mcrB promoter fusion to uidA with a AAA start site	This study
pJK026A	$\phi$ C31-attB vector with PmcRB promoter fusion to uidA	This study
pJK027A	$\phi$ C31-attB vector with PmcRB(tetO1) promoter fusion to uidA	This study
pJK028A	$\phi$ C31-attB vector with PmcRB(tetO3) promoter fusion to uidA	This study
pJK029A	$\phi$ C31-attB vector with PmcRB(tetO4) promoter fusion to uidA	This study
pJK031A	$\phi$ C31-attP vector with PmcRB(tetO1) promoter fusion to uidA	This study
pJK032A	$\phi$ C31-attP vector with PmcRB(tetO3) promoter fusion to uidA	This study
pJK033A	$\phi$ C31-attP vector with PmcRB(tetO4) promoter fusion to uidA	This study
pJK200	Fosmid vector encoding chloramphenicol and puromycin resistance with oriV, lattP and fC31-attB	This study
pWM321	<i>E. coli</i> /Methanosarcina shuttle vector	(Metcalf et al. 1997)
pWM357	Fosmid cloning vector	(Zhang et al. 2002)
pGK50A	Vector for testing gene essentially using PmcRB(tetO1), encodes kanamycin and puromycin resistance	This study
pGK51A	Vector for testing gene essentially using PmcRB(tetO3), encodes kanamycin and puromycin resistance	This study
pGK52A	Vector for testing gene essentially using PmcRB(tetO4), encodes kanamycin and puromycin resistance	This study
pGK50B	Vector for testing gene essentially using PmcRB(tetO1), encodes kanamycin and puromycin resistance, tetR gene is in opposite orientation to pGK50A	This study
pGK51B	Vector for testing gene essentially using PmcRB(tetO3), encodes kanamycin and puromycin resistance, tetR gene is in opposite orientation to pGK51A	This study
pGK52B	Vector for testing gene essentially using PmcRB(tetO4), encodes kanamycin and puromycin resistance, tetR gene is in opposite orientation to pGK52A	This study
pGK90	pGK050A-derived plasmid used for construction of WWM253	This study
pAB79	$\phi$ C31-attB vector with PmcRB(tetO1) fusion to uidA, can be used for construction of either transcriptional or translational fusions	This study

*PmcRB*(tetO4). The *PmcRB*(AAA) promoter, which has a three-base-pair mutation that eliminates the TATA box, was also constructed to demonstrate that transcription was being driven solely by *PmcRB*. Host strains that constitutively express the Tn10 *tetR* gene from the wild-type *mcrB* promoter were constructed to allow regulated expression from these hybrid promoters (Table 1). Some strains also carry the

$\phi$ C31-attB or  $\phi$ C31-attP site, along with an artificial operon that expresses both *tetR* and the  $\phi$ C31 *int* gene from the *PmcRB* promoter, to allow insertion of plasmids into the chromosome as described above.

To test the system, we fused each hybrid promoter to *uidA* and integrated the resulting plasmids into the *M. acetivorans* chromosome in single copy via site-specific recombination.

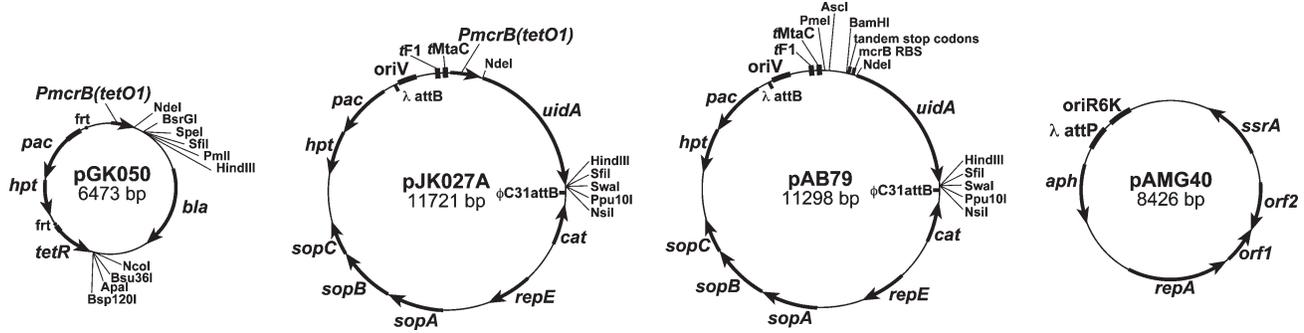


Figure 2. Structure of representative plasmids. Plasmids of the pGK050 series can be used to construct strains with Tet-dependent expression of *Methanosarcina* genes by “knocking in” a *PmcrB*(tetO) promoter at the normal chromosomal location of a gene of interest. To do this an appropriate region of homology upstream of the promoter to be deleted is cloned into one of the sites adjacent to *tetR*, while the gene of interest is cloned downstream of the *PmcrB*(tetO) promoter. Use of the *NdeI* site (CATATG) allows construction of in-frame translational fusions to the *PmcrB*(tetO) promoter (the underlined ATG within the *NdeI* site comprises the start codon of *mcrB*). Plasmids of the pJK027A series can integrate into the chromosome by  $\phi$ C31 site-specific recombination and are useful for construction of either translational *uidA* reporter gene fusions (by replacement of *PmcrB*(tetO) with a promoter of interest) or fusions of a gene of interest to a Tet-regulated promoter (by replacement of *uidA* with a gene of interest). Again, the *NdeI* site allows construction of in-frame translational fusions. Plasmid pAB79 can also integrate into the chromosome by  $\phi$ C31 site-specific recombination, but can be used to create either transcriptional or translational fusions to *uidA*. By cloning promoters of interest into the BamHI site, one can maintain the *mcrB* ribosome-binding-site (RBS) to allow efficient translation initiation of *uidA*; thus, expression of the reporter gene fusion is dependent only on transcription initiating within the cloned segment. Tandem translation stop codons are maintained in this case to prevent translational readthrough into the reporter gene. Alternatively, one can maintain the RBS from the gene of interest by cloning into the *NdeI* site, thus creating a translational fusion that requires both transcriptional and translational signals to be present in the cloned fragment. Plasmid pAMG40 carries the entire pC2A plasmid from *M. acetivorans* and is capable of autonomous replication in *Methanosarcina*. It can be used to retrofit non-replicating plasmids such as pAB79 or the pJK027A series by site-specific recombination between  $\lambda$ attB and  $\lambda$ attP. The resulting plasmid co-integrants are capable of autonomous replication in either *E. coli* or *Methanosarcina*. Additional plasmids similar to the ones shown here are presented in Table 2. Acronyms: *bla*,  $\beta$ -lactamase gene encoding ampicillin resistance; *tetR*, gene for the tetracycline-responsive repressor protein from Tn10; *hpt*, gene for hypoxanthine phosphoribosyl transferase; *pac*, puromycin acetyltransferase gene encoding resistance to puromycin; FRT, recognition site for the F1p site-specific recombinase; *uidA*, gene encoding  $\beta$ -glucuronidase; *cat*, chloramphenicol acetyltransferase gene encoding resistance to chloramphenicol; *repE*, gene encoding the replication initiation protein from the *E. coli* F plasmid; *sopA*, *sopB* and *sopC*, genes encoding the plasmid partitioning system of the *E. coli* F plasmid;  $\lambda$ attP and  $\lambda$ attB, the recognition sites for the phage  $\lambda$  Int site-specific recombinase; tF1 and tMtaC, putative transcriptional terminators from the *E. coli* phage F1 and *M. acetivorans mtaCB1* operon, respectively.

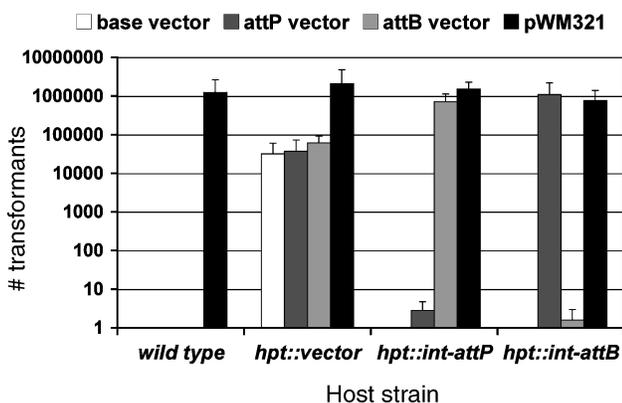


Figure 3. Transformation efficiencies in *Methanosarcina* using the  $\phi$ C31 integrase-mediated integration. Various *Methanosarcina* strains were transformed to Pur<sup>R</sup> with 2  $\mu$ g of the indicated plasmid DNA and the number of colonies obtained was quantified. The presence of  $\phi$ C31 Int recombination sites (attB or attP) are indicated. Results shown are means of least three trials. Host strains used were WWM1 (wild-type), WWM19 (*hpt::vector*), WWM73 (*hpt::int-attP*) and WWM75 (*hpt::int-attB*). Plasmids used were pAMG18 (base vector), pAMG44 (attP vector), pAMG45 (attB vector) and pWM321 (autonomous plasmid vector).

We then measured  $\beta$ -glucuronidase activity after growth in media with and without tetracycline (Table 3). In the absence of tetracycline,  $\beta$ -glucuronidase activity was below the limit of detection in strains that express *uidA* from the *PmcrB*(tetO1), *PmcrB*(tetO3) and *PmcrB*(tetO4) promoters, suggesting that TetR binding prevents transcription from the hybrid promoters. The level of expression was significantly lower than that observed from the *PmcrB*(AAA) promoter in which the TATA box was intentionally destroyed. Thus, the TetR-binding sites prevent even basal rates of transcription in these strains. Addition of tetracycline to the cultures resulted in activities that ranged from high to low (Table 3). (Tetracycline did not change the growth rate of wild-type strains (data not shown).) These data indicate that the TetR-binding sites alter the efficiency of the hybrid promoters, lowering the induced expression by a factor of two to 35, relative to the wild-type *PmcrB* promoter. Nevertheless, each of the resulting promoters was tightly regulated by the presence or absence of tetracycline.

Additional experiments were performed to assess the kinetics of induction and to examine whether expression could be tuned by adding different concentrations of tetracycline (Figure 5). At tetracycline concentrations greater than 33  $\mu$ g ml<sup>-1</sup> induction was essentially complete; however, at lower concentrations of tetracycline (< 10  $\mu$ g ml<sup>-1</sup>) a graded response was

**Minimal *pmcrB***GCATGCTTCATTTATCGGAGAACAC **AAAAGATTTAAGT**ACCTTCTAAACGAATGAGAT TTCATTGGGAATAGTGGACACTCGAG**Minimal *pmcrB*(AAA)**GCATGCTTCATTTATCGGAGAACAC **AAAAGAAAAAAGT**ACCTTCTAAACGAATGAGAT TTCATTGGGAATAGTGGACACTCGAG**Minimal *pmcrB*(tetO1)**GCATGCTTCATTTATCGGAGAACAC **AAAAGATTTAAGT**ACCCTATCAGTGATAGAGA TTCATTGGGAATAGTGGACACTCGAG**Minimal *pmcrB*(tetO3)**GCATGCTTCATTTATCGGAGAACAC **AAAAGATTTAAGT**ACCCTATCAGTGATAGAGA TTCCCTATCAGTGATAGAGA CTCTGAG**Minimal *pmcrB*(tetO4)**GCATGCTTCATTTATCGGAGAACAC **AAAAGATTTAAGT**ACCTTCTAAACGAATGAGAT TTCCCTATCAGTGATAGAGA CTCTGAG

Figure 4. Nucleotide sequence of the *mcrB* promoter and mutated derivatives. The nucleotide sequence of the minimal *mcrB* promoter from *Methanosarcina barkeri* is shown on the top line. The putative BRE is shown in bold text, the putative TATA box is underlined and the experimentally verified transcription start site (Allmansberger et al. 1989) is underlined in bold text. The following lines show the mutated derivatives that were modified to include the *tetR*-binding site (double underlined) at various positions within the promoter.

observed. Expression was not observed when tetracycline was added at less than 0.5  $\mu\text{g ml}^{-1}$ . Time course experiments showed that the response to tetracycline was rapid, with measurable GUS activity being observed within 30 min of the addition of the inducer. However, full expression was not achieved until the cultures reached stationary phase, approximately 48 hours later.

We examined regulation by the hybrid promoters when they were carried on multi-copy plasmids in *Methanosarcina*. To do this we constructed pAMG40, a bifunctional plasmid that replicates in both *E. coli* and *Methanosarcina* (Figure 2). The plasmid carries the phage  $\lambda$ -attP site allowing  $\lambda$ -integrase-mediated site-specific recombination with the fosmid vectors described above. Thus, fosmid:pAM40 co-integrants can be con-

structed by in vitro recombination using commercially available recombinase preparations. This allows facile conversion of the non-replicating integration plasmids described above into autonomous *Methanosarcina* plasmids. (These experiments are conducted in strains that lack the  $\phi\text{C31-int}$  gene to

Table 3. Gus activity of *PmcrB::uidA* fusions and derivatives.

Promoter	Tet	Gus activity (mU)	
		Chromosome <sup>1</sup>	Plasmid <sup>2</sup>
No <i>uidA</i> fusion	-	< 0.4	nd
	+	< 0.4	nd
<i>PmcrB::uidA</i>	-	1601.1 $\pm$ 185.9	1760.4 $\pm$ 497.5
	+	1502.7 $\pm$ 132.0	1777.0 $\pm$ 389.1
<i>PmcrB</i> (AAA):: <i>uidA</i>	-	1.8 $\pm$ 0.3	26.9 $\pm$ 3.9
	+	1.3 $\pm$ 0.2	22.3 $\pm$ 4.6
<i>PmcrB</i> (tetO1):: <i>uidA</i>	-	< 0.4	< 0.4
	+	792.8 $\pm$ 20.0	2598.0 $\pm$ 491.2
<i>PmcrB</i> (tetO3):: <i>uidA</i>	-	< 0.4	< 0.4
	+	45.2 $\pm$ 8.9	387.0 $\pm$ 81.1
<i>PmcrB</i> (tetO4):: <i>uidA</i>	-	< 0.4	< 0.4
	+	385.4 $\pm$ 36.3	997.0 $\pm$ 163.1

<sup>1</sup> Strains assayed were WWM73 and single-copy integrants of pJK200-*PmcrB::uidA*, pJK200-*PmcrB*(AAA)::*uidA*, pJK200-*PmcrB*(tetO1)::*uidA*, pJK200-*PmcrB*(tetO3)::*uidA* and pJK200-*PmcrB*(tetO4)::*uidA* into WWM73.

<sup>2</sup> Strains assayed were WWM60 and WWM60 carrying autonomously replicating plasmid co-integrants pAMG40 with pJK200-*PmcrB::uidA*, pJK200-*PmcrB*(AAA)::*uidA*, pJK200-*PmcrB*(tetO1)::*uidA*, pJK200-*PmcrB*(tetO3)::*uidA* and pJK200-*PmcrB*(tetO4)::*uidA*.

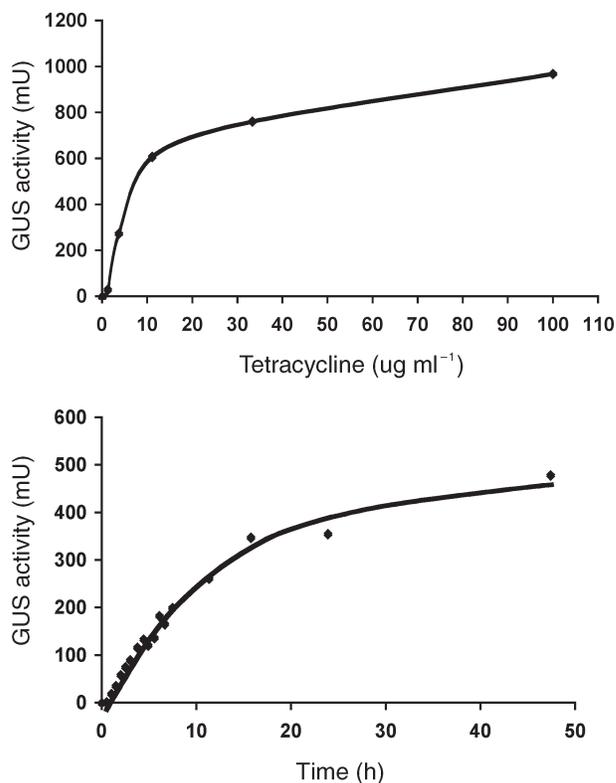


Figure 5. Dose-response and time course of tetracycline-dependent gene expression in *Methanosarcina*. Panel A, Plasmid pJK027A was integrated into the chromosome of strain WWM73 and the resulting strain was grown in the presence of various tetracycline concentrations to mid-exponential phase before assaying GUS activity as described. Panel B, The same strain was grown without tetracycline until the culture reached early exponential phase. Tetracycline was then added at a concentration of 100  $\mu\text{g ml}^{-1}$ . At various times, samples were withdrawn and assayed for GUS activity.

avoid recombination of the multi-copy plasmid into the chromosome.)

Tet-inducible expression of the hybrid promoters carried on autonomous plasmids was 3- to 8-fold higher than that observed when the plasmids were inserted into the chromosome, (Table 3). These values are consistent with the copy number of the pC2A replicon used in pAMG40, which has been estimated at approximately six copies per cell (Sowers and Gunsalus 1988). The more highly expressed promoters showed less of an increase, relative to the chromosomal insertions, than the promoters with lower expression, suggesting that other transcriptional factors may be limiting at high levels of expression.

#### Use of tet-regulated promoters to test gene essentiality

The exceptionally stringent regulation of the hybrid promoters allows their use to test gene essentiality. To facilitate such studies, we constructed the pGK050 series of plasmids (Table 2), which contain selectable and counter-selectable markers, one of the hybrid promoters and a copy of the *PmcrB::tetR* gene (Figure 2). To use these plasmids, the gene of interest is fused to the appropriate *PmcrB(tetO)* promoter (chosen based on the levels of expression of the native gene). A region of homology upstream of the target gene's promoter is then cloned on the other side of the selectable/counter selectable markers. The resulting plasmid is linearized and recombined onto the chromosome, resulting in replacement of the native promoter with the Tet-regulated promoter. This transformation is performed in the presence of tetracycline to allow expression of the presumptive essential gene. We typically use

host strains that carry an additional copy of *tetR* gene inserted into the chromosomal *hpt* locus. This greatly reduces the probability of obtaining constitutive, *tetR*-minus mutants that can confuse the results of the test. Once the strain is verified, growth studies in the presence or absence of tetracycline can be performed to assess whether the cells are viable when the target gene is not expressed (i.e., in the absence of tetracycline). The presence of the counter-selectable marker, which is flanked by recognition sites for the Flp site-specific recombination system, allows removal of the selectable marker should subsequent experiments requiring the puromycin selection be desired (Rother and Metcalf 2005).

To test this system, we constructed an *M. barkeri* strain with the *mcrBDCGA* operon under the control of the *PmcrB(tetO1)* promoter. The resulting strain grew well on solid medium with the methanol as a growth substrate, so long as tetracycline was included. However, no growth was observed in the absence of tetracycline (Figure 6). Similar results were obtained in liquid media containing acetate, H<sub>2</sub>/CO<sub>2</sub> or H<sub>2</sub>/CO<sub>2</sub>/methanol as growth substrates, indicating that the *mcr* operon is essential for growth on these substrates as well (data not shown).

#### Discussion

The  $\phi$ C31-based site-specific recombination system reported here represents a substantial improvement on previous methods that employ homologous recombination to catalyze stable integration of heterologous DNA into the chromosome of *Methanosarcina* (Pritchett et al. 2004). Not only is the new system at least 30-fold more efficient at generating recombinants, it also reduces by half the time needed to create strains. The previously used method required a preliminary integration step, followed by a segregation step to produce stable recombinants carrying the DNA of interest. Because growth of *Methanosarcina* colonies on solid medium requires about 14 days, this method takes a total of about two months because of the need to purify transformants by streaking on solid medium after each step. Thus, utilization of the  $\phi$ C31 system saves a full month over the earlier method. Further, the  $\phi$ C31 integration is unidirectional, providing stability of the insert. In the studies reported here puromycin selection was not maintained after initial isolation of the strain, yet the integrated plasmids were never lost. This system should prove useful for a variety of applications such as single-copy mutant complementation studies, thus relieving problems that occasionally occur when performing episomal complementation of mutants, especially when membrane protein complexes are encoded on the plasmid (Meuer et al. 2002). It is also particularly useful for the construction of promoter gene fusions in *Methanosarcina*. In a recent study we used this system to place a series of reporter gene fusions into a variety of mutant backgrounds. In this study sixty-eight strains were constructed in a short time with a minimum of effort (Bose and Metcalf 2007). Given the labor and time required, such a study would not have been possible without the efficient and rapid  $\phi$ C31 system.

The observation that many *Methanosarcina* genes utilize start codons other than ATG raises potential problems in com-

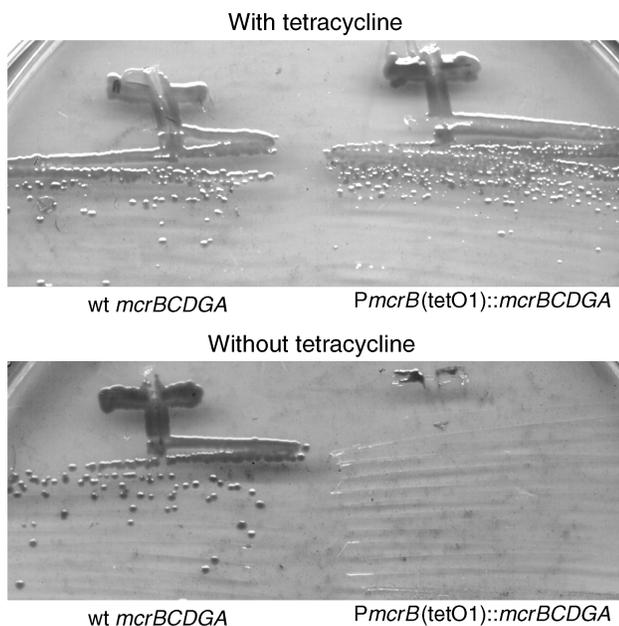


Figure 6. Essentiality of the *mcr* operon in *Methanosarcina barkeri*. WWM155 and WWM235 were streaked on HS-methanol agar in the presence (100  $\mu$ g ml<sup>-1</sup>) or absence of tetracycline. Growth of the *PmcrB(tetO1)::mcrBCDGA* only in the presence of tetracycline indicates that the *mcr* operon is essential.

paring the results obtained using translational reporter genes fusions. For example, *mcrB* uses a GTG start site, while *frhA* (encoding a hydrogenase subunit) and *pta* (encoding phosphotransacetylase) use TTG start sites (Bokranz and Klein 1987, Latimer and Ferry 1993, Vaupel and Thauer 1998). At least one gene, the *repA* gene of the pC2A plasmid, is predicted to utilize a CTG translation start (Metcalf et al. 1997). Thus, we were interested in determining the relative efficiency of different start codons in *Methanosarcina*. Our data indicate that GTG, TTG, and even CTG are efficiently used in *Methanosarcina*, albeit at lower levels than ATG. In cultured monkey CV1 cells TTG and GTG start codons are used poorly, if at all. Instead, translation initiation in this eukaryote occurs efficiently using ACG, and less efficiently using CTG, ATC, ATT and ATA (Peabody 1989). Thus, although archaeal translation initiation is known to share features in common with both bacteria and eukarya (Londei 2005), our data indicate that choice of initiation codon in *Methanosarcina* is much more similar to bacteria.

Tightly regulated gene expression systems are among the most needed genetic tools in research with archaea (Allers and Mevarech 2005, Rother et al. 2005). Existing expression systems in methanoarchaea are based on fusions of the gene of interest to a catabolic promoter involved, e.g., in methanol, acetate utilization or assimilation of nitrogenous compounds (Apolinario et al. 2005, Lei et al. 2005, Rother et al. 2005). Expression of these fusions is minimized during growth on other substrates and can be induced by switching the culture to the respective catabolic substrate. Thus, expression of the target gene requires growth on a particular substrate, which can be problematic if one is interested in the role of a particular gene under a variety of conditions. We chose to adapt the tetO/TetR system from *E. coli* because, first, it is well characterized (reviewed in Hillen and Berens (1994) and, second, methanogenic archaea are intrinsically insensitive to tetracycline (Böck and Kandler 1985, Possot et al. 1988). The regulation of the hybrid promoters that we constructed is especially tight and the expression of both homologous and heterologous genes can be induced quickly, several thousand-fold, and independently of the growth phase of the host or the energy substrate utilized. Furthermore, our data suggest that tuning of expression is feasible by titration with tetracycline. However, it remains to be shown if this regulation is dose-dependent for the whole *Methanosarcina* population, as is the case for tetO/TetR systems in bacteria and eukaryotes, or an auto-catalytic induction of expression due to active uptake of the inducer, as is the case for Plac- and Para-dependent gene expression (Novick and Weiner 1957, Morgan-Kiss et al. 2002). With the Pmcr(tetO)/TetR system established for *Methanosarcina* it seems feasible now to overproduce enzymes in a catalytically active form where other host/over-expression systems have resulted in partially inactive protein (Roberts et al. 1989, Sauer et al. 1997, Sauer and Thauer Rudolf 1998, Loke et al. 2000). Furthermore, even toxic genes can probably be overproduced because of the tight repression of the hybrid promoter in the absence of tetracycline. The TetR system allows, for the first time, testing gene essentiality in

*Methanosarcina* in a positive, rather than a negative manner. This is in stark contrast to commonly used methods that rely on statistical evidence such as absence of transformants (Stathopoulos et al. 2001).

Finally, this study illustrates the usefulness of  $\phi$ C31 integrase-mediated integration systems and the tetO/TetR mediated system of inducible gene expression in *Methanosarcina*. Previous studies have demonstrated their functionality in both bacteria and eukarya. That they function in methanogenic archaea, while not surprising, indicates that they could probably be adapted to other archaeal species where genetic systems exist.

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### Supplementary material

File 1. *Methanosarcina* Locus pWM357, 8688 bp. Available at:

<http://archaea.ws/archive/supplementary/2-193.FileS1.pdf>

File 2. *Methanosarcina* pAMG27 3629 bp. Available at:

<http://archaea.ws/archive/supplementary/2-193.FileS2.pdf>