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pNEB193-derived suicide plasmids for gene deletion and protein expression in the methane-producing archaeon, *Methanosarcina acetivorans*

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Abstract

Gene deletion and protein expression are cornerstone procedures for studying metabolism in any organism, including methane-producing archaea (methanogens). Methanogens produce coenzymes and cofactors not found in most bacteria, therefore it is sometimes necessary to express and purify methanogen proteins from the natural host. Protein expression in the native organism is also useful when studying post-translational modifications and their effect on gene expression or enzyme activity. We have created several new suicide plasmids to complement existing genetic tools for use in the methanogen, *Methanosarcina acetivorans*. The new plasmids are derived from the commercially available *E. coli* plasmid, pNEB193, and cannot replicate autonomously in methanogens. The designed plasmids facilitate markerless gene deletion, gene transcription, protein expression, and purification of proteins with cleavable affinity tags from the methanogen, *Methanosarcina acetivorans*.

1. Introduction

Genetic methods for *Methanosarcina* species are well developed, and making mutations on the chromosome is a routine procedure in several laboratories [1–5]. We sought to increase the ease-of-use for these tools to facilitate cloning, protein expression, and molecular biology experiments. The plasmid tools we have created complement existing methods and expand the repertoire of in vivo experiments possible in methanogens. Of particular need is the ability to express tagged proteins in methanogens to facilitate protein purification from the native host.

Methanogens survive by reducing carbon substrates to methane gas in a process called methanogenesis [6]. They employ unique enzymes and cofactors to activate carbon for reduction, and simultaneously generate a transmembrane ion gradient that is used for ATP synthesis [7, 8]. Unusual cofactors used in methanogens include coenzyme M, coenzyme B, methanopterins, methanophenazine, dimethylbenzimidazolyl cobamide, and deazaflavin F_{420} [9–25]. Because of these unusual cofactors, it may be difficult or impossible to express some methanogen proteins in heterologous hosts that do not produce these cofactors. If

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cofactor binding is essential for proper protein folding, the absence of the cofactor may result in misfolded and/or insoluble protein. If the protein does fold properly in a heterologous host, it is possible that host cofactors may bind in the active site in place of the native cofactor. For instance, dimethylbenzimidazolyl cobamide is structurally similar, though not identical to cobalamin [26–28]. The methanogen methanol:corrinoid methyltransferase, MtaB, and the corrinoid protein, MtaC, from *Methanosarcina barkeri*, have been purified from *E. coli* and biochemically characterized [29–31]. MtaB and MtaC expressed in *E. coli* are insoluble, and must be refolded in vitro after purification. As a result, MtaC is devoid of cofactor and must be reconstituted with the non-native corrin cofactor, hydroxycobalamin. While heterologously expressed, refolded, and reconstituted protein can be suitable for biochemical characterization, these treatments introduce the possibility of producing structural artefacts that can inhibit crystal formation. As such, overexpression of proteins in the native organism can be desirable to purify soluble protein populated with the biologically relevant cofactor. The crystal structure of the MtaBC complex was successfully obtained using protein purified from *M. barkeri* [32].

Dimethylbenzimidazolyl cobamide is not the only exotic cofactor found in methanogens. Coenzyme F_{420} is a deazaflavin, and structurally similar to flavin mononucleotide (FMN) in *E. coli* [19, 33–36]. To our knowledge, no predicted flavin-binding proteins from methanogens has been heterologously expressed or crystallized to date. One reason for the paucity of methanogen flavoprotein structures could perhaps be because annotated flavin adenine dinucleotide (FAD) or FMN binding sites may in fact be F_{420} binding sites. Therefore *E. coli* flavins may not be able to bind correctly in F_{420} binding site, resulting in unstable or misfolded protein. Methanogens also express many proteins with catalytic or structural iron/sulfur clusters, which do not have homologs in *E. coli* [37–45]. Therefore, expression of iron/sulfur cluster proteins in *E. coli* runs the risk of producing insoluble or misfolded protein, which may or may not be able to be reconstituted in vitro with Fe^{2+} and S^{2-} [46].

Several methanogen proteins which do not require cofactors have been successfully expressed from *E. coli*, such as histone-like proteins, glutamine synthetase GlnK, and CRISPR Cas6 [47–50]. However, in some circumstances though proteins are not anticipated to require a cofactor or iron/sulfur cluster, expression of methanogen proteins in *E. coli* can still be challenging due to differences in codon usage between the two organisms [51–54]. Codon usage is significantly different between *E. coli* and methanogens. Translation of methanogen proteins can be accomplished using *E. coli* expression strains engineered to produce rare codons, however the yields can be low [55–57]. The yield of heterologously expressed methanogen protein can be increased by codon optimizing the nucleotide sequence for *E. coli* [55, 57]. Synthesis of codon-optimized genes is more expensive than traditional cloning involving PCR amplification of the gene of interest. Taking into account the high proportion of methanogen proteins with iron/sulfur clusters and unique cofactors, we perceived a need for a wider array of molecular tools for protein expression and purification in methanogens.

To address the need for plasmids that can be used to express and purify protein from methanogens, we designed new suicide plasmids based on the features of pMP44 and

pJK026A [58, 59]. pMP44 is useful for markerless deletion of genes using homologous recombination [58]. However, pMP44 replicates in the *E. coli* host at a relatively low-copy number and must be propagated in a *pir*$^{+}$ strain [60, 61]. Plasmid pJK026A and its derivatives can be used for inserting DNA at a φC31 phage *att* site which has been added to the chromosome [59]. It is useful for expressing protein in *Methanosarcina*, or for studying transcription and translational fusions [2]. pJK026A family plasmids are 11.7 Kb, and must be purified from a *trfA*$^{+}$ *E. coli* strain [62]. The plasmid sizes, low copy number, and need for separate *E. coli* host strains, are attributes that can present technical challenges during cloning. We wanted to determine if the features of pMP44 and pJK026A could be used to create smaller plasmids that are suitable for high-copy replication in DH5α or DH10β *E. coli* hosts. The new suicide plasmids are designed to 1) use conventional, commercially-available *E. coli* hosts, 2) simplify and speed up the cloning process, and 3) combine features in a multifunctional plasmid that can stably integrate onto the *M. acetivorans* chromosome and be used for in vivo protein expression and purification via Strep-Tag II and histidine affinity tags [63–65].

2. Materials and Methods

2.1 Growth of cultures

E. coli was grown in Lysis Broth (LB) at 37°C with shaking [66]. *M. acetivorans* strains were grown at 35°C in HS medium as described [67]. Table 1 lists all the *E. coli* and *M. acetivorans* strains used in this study. The following additions were added as required (final concentration): ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), chloramphenicol (8 or 35 μg ml^{-1}), rhamnose (10 mM), histidine (0.1 mM), puromycin (2 μg ml^{-1}), 8-aza-diaminopurine (8-ADP) (20 μg ml^{-1}), trimethylamine (50 mM), methanol (125 mM), and acetate (40 or 120 mM).

2.2 DNA techniques and cloning procedures

PCR Primers and DNA sequences in Table 2 were designed using Vector NTI software (Life Technologies Corporation, Grand Island, NY). Genes, oligos, and multiple cloning sites were synthesized commercially by Integrated DNA Technologies (IDT, Coralville, IA) and Life Technologies Corporation (Grand Island, NY). Various PCR techniques were employed during the course of this work, including overlap extension and site-directed mutagenesis [68, 69]. For all PCR amplifications, Phusion Flash PCR Master Mix was used as a source of proofreading DNA polymerase (Life Technologies Corporation (Grand Island, NY)). DNA purification was carried out using Wizard kits from Promega (Madison, WI). DNA fragments were joined using T4 DNA ligase (New England Biolabs, Ipswich, MA) or GeneArt kits (Life Technologies Corporation (Grand Island, NY)). Restriction enzymes (*AscI, BamHI, NdeI, NcoI, EcoRI, SphI, XbaI*) were purchased from New England Biolabs (Ipswich, MA). All plasmids were sequenced by Eurofins Operon MWG (Huntsville, AL).

2.3 Transformation

Plasmids used and created in this study are listed in Table 3. *E. coli* was transformed by electroporation and plated onto LB agar plates (1.5% w/v agar) containing the appropriate antibiotic [70]. *M. acetivorans* was transformed using the liposome-mediated transformation

method [71]. After transformation and recovery, *M. acetivorans* cells were plated on HS medium with 50 mM trimethylamine as carbon source solidified with 1.4% agar and incubated in Wolfe incubators (Coy Laboratory Products, Grass Lake, MI) under premixed 20% CO_2/79.9% N_2/0.1% H_2S atmosphere (Matheson).

2.4 Western Blot Analysis

Sample protein concentration was determined using the Coomassie Plus Protein Assay Reagent (Life Technologies Corporation (Grand Island, NY)). Samples were diluted with 6X Cracking Buffer (348 mM Tris pH 6.8, 349 mM SDS, 600 mM DTT, 4.1 mM glycerol, 180 μM bromophenol blue), boiled for 10 minutes, and 2 μg each sample were loaded per lane on a 12% sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad, Hercules, CA). Three microliters of Precision Plus Protein Dual Color Standards (Bio-Rad, Hercules, CA) and 1.5 μL of Precision Plus Protein WesternC Standards (Bio-Rad, Hercules, CA) were used as markers. Proteins were separated at 15 mA per gel for 30 minutes and 30 mA per gel for 45 minutes. Proteins were transferred to a polyvinylidene difluoride membrane (PVDF) (Bio-Rad, Hercules, CA) for 1 hour at 100 V in transfer buffer (25 mM Tris, 192 mM glycine, 15% methanol). The membrane was blocked with 25 mL 5% nonfat dry milk in Tris-buffered saline (137 mM NaCl, 20 mM Tris pH 7.6) with 0.1% TWEEN 20 (TBST) overnight and probed with a 1:4000 dilution of Strep-Tag II Antibody, HRP Conjugate (Novagen, EMD Millipore, Temecula, CA) in 20 mL of Blocking Solution. Strep-tagged protein was detected with Pierce ECL Western Blotting Substrate (Life Technologies Corporation (Grand Island, NY)).

2.5 β-glucuronidase enzyme assays

Cell extract was assayed for β-glucuronidase activity as described [59]. Briefly, 10 ml exponential phase cultures of strains listed in Table 1 were harvested by centrifugation in a TX-750 Swinging Bucket Rotor with 15 mL conical tube adapters at 4031 × *g* for 3 minutes at room temperature. Cells were resuspended in 200 μl of 50 mM Tris-Cl, 1 mM DTT pH 8.0 buffer, followed by the addition of 1 u of DNaseI (Life Technologies Corporation (Grand Island, NY)) and Halt Protease Inhibitor Cocktail, EDTA-Free (Life Technologies Corporation (Grand Island, NY)) to a final concentration of 1X. Cells were lysed on ice for 10 minutes, and insoluble cell debris was removed by centrifugation in F21–48×1.5/2.0 rotor at 14000 × *g* for 10 minutes at room temperature. Cleavage of *p*-nitrophenyl glucuronide to *p*-nitrophenol was detected by increased absorbance at 415 nm in a Tecan Sunrise plate spectrophotometer (Tecan US, Inc., Morrisville NC). The extinction coefficient of *p*-nitrophenol was determined in Solid 96 Well Plates (Fisher catalog #21-377-205) with a path length of 0.5 cm at 415 nm. Protein concentration was measured using the Coomassie Plus Protein Assay Reagent (Life Technologies Corporation (Grand Island, NY)).

3. Results

3.1 pNB723 plasmid design

We created a high-copy plasmid, pNB723, for markerless deletion of *Methanosarcina* genes to circumvent the need to use *pir*$^+$ *E. coli* hosts (Figures 1 and S1). To construct pNB723, we used pNEB193 as the *E. coli* plasmid scaffold (New England Biolabs, Ipswich, MA).

pNEB193 is a small, high-copy, commercially available vector with a pUC19 origin of replication, a bleomycin (ampicillin) resistance cassette, and a P_{T7} *lacZ* cassette for blue-white selection of plasmids containing inserts at the multiple cloning site. To select *Methanosarcina* strains which have recombined the plasmid onto the chromosome, we added the *pac* (puromycin acetyltransferase) gene at the unique *XbaI* and *SphI* restriction sites on pNEB193. We optimized the *pac* codons for expression in *Methanosarcina*, thereby lowering the %GC content from 73.1% to 48.5%, and eliminating interference in sequencing reactions that can occur when plasmids contain high %GC stretches (Figure S2). The optimized *pac* gene was amplified from pMS86 using oligos oNB115 and oNB116, which added a *XbaI* restriction site at the 5' end of the gene, and tandem *SpeI* and *SphI* restriction sites at the 3' end of the gene. The resulting plasmid carrying a promotorless optimized *pac* gene is pNB721.

The *Methanococcus voltae PmcrB* promoter from pMP44 was cloned upstream of the *pac* gene at the *XbaI* restriction site, creating plasmid pNB722. The $PmcrB_{(M.\ voltae)}$ promoter will constitutively express the *pac* gene in *Methanosarcinales*. $PmcrB_{(M.\ voltae)}$ was cloned from pMP44 using oligos oNB128 and oNB129, which added *XbaI* sites at each end of the gene. Orientation of the promoter was verified by DNA sequencing to ensure that the *pac* gene will be expressed in the host strain.

Finally, we cloned a hypoxanthine phosphoribosyltransferase gene (*hpt*) at the *SpeI* and *SphI* restriction sites in pNB722 so that the resulting plasmid, pNB723, expresses the *hpt* gene in an operon with the *pac* gene. The *hpt* gene is a counterselection marker that can be used to create a markerless gene deletion when transformants are plated on the purine analog, 8-aza-diaminopurine (8-ADP) [58]. The *hpt* gene was also codon-optimized for expression in *Methanosarcina*, which resulted in lowering the %GC content from 47.6% to 36.7% (Figure S3). The optimized *hpt* gene was amplified from pMS66 using oligos oNB106 and oNB127, which added a *XbaI* site at the 5' and at the 3' ends of the gene. Directionality of the *hpt* gene was verified by DNA sequencing. The resulting pNB723 plasmid has unique *NdeI* and *BamHI* sites, and two *AscI* restriction sites, which can be used to clone DNA sequences for deletion of genes in *Methanosarcina*.

3.2 Deletion of MA4421 using pNB723

To demonstrate that pNB723 functions as designed, we used it to delete the *MA4421* prenyl reductase gene from the *M. acetivorans* chromosome (Figure 2). For the plasmid validation purposes here, the gene to be deleted was expected to be nonessential. The DNA sequence 5' upstream of the *MA4421* gene was amplified using primers oNB250 and oNB252. The 3' downstream DNA sequence was amplified using primers oNB251 and oNB253. The 5' and 3' sequences were fused using oligos oNB311 and oNB312, and cloned into the pNB723 plasmid at the *AscI* restriction site, resulting in plasmid pALD1 (Figure S4). Plasmid pALD1 was transformed into *M. acetivorans* strain NB34 using liposomes, and puromycin as a selection agent. The puromycin-resistant colonies were streaked for isolation, grown in liquid medium without puromycin, and plated onto agar containing 8-ADP to counterselect for the *hpt* gene. The resulting 8-ADP-resistant colonies were streaked for isolation, and grown in liquid medium without puromycin or 8-ADP. Markerless deletion of the *MA4421*

gene was confirmed by PCR amplifying the *MA4421* deletion junctions from genomic DNA using the oNB274, oNB318 and oNB319 oligos (Figure 2). In this PCR strategy, the screening oligos do not anneal to the 5' upstream or 3' downstream DNA sequences that were used to construct the pALD1 deletion plasmid. *Methanosarcina* cells can carry several copies of the chromosome, and PCR amplification for the deleted gene is essential to ensure that all copies of the gene have been deleted [72]. In addition, plasmids may integrate in unpredictable ways if there is a region of low complexity or if the gene is essential. Surviving cells may, for instance, create large deletions, amplifications, or insertions to preserve essential gene function while also generating a false-positive in a PCR screen. As additional measures to confirm strain identity, genome resequencing and/or Southern blots using probes specific for the deleted gene, for flanking genomic regions, or for *pac* or *bla* (to verify plasmid insertion at the expected location) is also indicated (Figure S5).

3.3 pNB730 plasmid design

We created a pNEB193-derived plasmid for expression of tagged protein in *Methanosarcina*. Using oligos oNB151 and oNB152, we removed the *SpeI* restriction site, creating plasmid pNB724. pNB724 was amplified using oligos oNB110 and oNB111, which creates *SpeI* restriction sites at the 5' and 3' ends of the linear amplification product. To insert the φC31 phage *attB* attachment site that allows the plasmid to recombine with the φC31 *attP* site on the *M. acetivorans* NB34 chromosome, we amplified the φC31 phage *attB* site from pJK026A using oligos oNB117 and oNB118, which creates *XbaI* restriction sites at the 5' and 3' ends of the amplification product. The *XbaI*-digested *attB* fragment was ligated into the *SpeI*-digested pNB724 amplicon to create pNB727. pNB727 was verified by DNA sequencing.

Next, we designed an expression cassette with multiple restriction sites to facilitate cloning (Figure 3). The cassette, encoded on plasmid pNB716, contains the *PmcrB* promoter from pJK026A and a multiple cloning site (MCS) flanked by sequences encoding the Strep-Tag II peptide (strep, WSHPQFEK) and histidine tags (his, HHHHHHHH). The Strep-Tag II peptide was codon optimized for expression in *M. acetivorans* (Figure 3, orange shaded sequences). The 5' and 3' tag sequences were not identical so as to prevent homologous recombination that would result in loss of the MCS or of the gene to be expressed. The expression cassette was designed such that cloning a gene into the *NdeI* site results in expression of native protein. Cloning the gene into the *NcoI* site results in protein with an amino-terminal his-strep tag. Carboxy-tagged protein can be expressed by removing the stop codon from the gene and cloning into the *BamHI, ApaI,* or *NruI* restriction sites. Therefore this expression cassette can be used to express native, amino-tagged, carboxy-tagged, or dual-tagged protein depending on the restriction sites used. A strong translational stop signal was added after the 3' his-strep tag sequence by introducing four stop codons within a 20 bp region. The expression cassette was amplified from pNB716 using oligos oNB183 and oNB184 and digested with *XbaI* restriction enzyme.

pNB727 was amplified using oligos oNB130 and oNB131, resulting in a linearized amplicon containing *SpeI* restriction sites at the 5' and 3' termini. The pNB727 amplicon was digested with *SpeI*, then ligated with the *XbaI*-digested expression cassette from

pNB716, to create the plasmid pNB729. Finally, oligos oNB185 and oNB186 were used to amplify pNB729 and remove the *BamHI* restriction site upstream of the *pac* expression cassette. The resulting plasmid, pNB730, contains unique *NdeI, NcoI, BamHI, ApaI,* and *NruI* restriction sites for cloning genes into the expression cassette multiple cloning site. pNB730 was verified by DNA sequencing (Figure S6).

3.4 Native and tagged expression of uidA using pNB730

We used the β-glucuronidase (*uidA)* gene to measure tagged and untagged protein expression in *M. acetivorans* from the expression cassette we created (Figure 4). The *uidA* gene was amplified from pJK026A using oligos oNB369 and oNB371 and cloned into the *NdeI* and *BamHI* sites of pNB730 to create plasmid pSK1 (expresses native UidA) (Figure S7). The *uidA* gene was also amplified from pJK026A using oligos oNB370 and oNB372, and cloned into the *NcoI* and *BamHI* sites to create plasmid pSK2 (expresses dual-tagged UidA) (Figure S8). Plasmids pSK1 and pSK2 were transformed into *M. acetivorans* strain NB34. Cells which had recombined the plasmid onto the chromosome at the φC31 *attP* site were selected using puromycin. Puromycin-resistant colonies were streaked for isolation, grown in liquid medium without antibiotic, and screened by PCR.

To screen for integration at the *attP* site, oligos “C31 screen-all#1”, “C31 screen C2A #1”, “C31 screen pJK200#1”, and oNB317 were used in a four-oligo PCR reaction with genomic DNA [59]. In this four-oligo PCR amplification, genomic DNA from strains which have integrated a single copy of pNB730-derived plasmids will produce amplicons of 740 and 471 bp. A 301bp band is amplified by plasmid alone or if multiple copies of the pNB730-derived plasmid has integrated at the φC31 *attP* site. Parental genomic DNA template will result in amplification of a 910 bp fragment. Using this screen, we verified the creation of strains NB231 (Δ*hpt*::φC31 *int*, *att*:pSK1) and NB232 (Δ*hpt*::φC31 *int*, *att*:pSK2), which had recombined the respective plasmid at the φC31 *attP* site on the chromosome. Plasmid integration was also confirmed by Southern blot using the *uidA* gene as a probe (Figure S9).

Next, we verified expression of UidA enzyme from the integrated pSK1 or pSK2 plasmids (Figure 4). To verify expression of tagged protein, we analyzed cell extract from strains NB231 (*att*:pSK1), NB232 (*att*:pSK2) by Western blot using anti-strep-tag antibodies. The parent extract was used as a negative control, and the positive control was cell extract from strain NB75, which expresses strepHdrD2 (37 kDa) from an integrated copy of the pJK026A-derived plasmid, pNB665. The cell extract from strains that expressed tagged protein had a single strep-tagged protein band at the expected size of 71 kDa, whereas the parent extract and NB231 (*att*:pSK1, which expresses untagged UidA protein), had no visible bands. We also added 0.1 mM histidine to cultures to determine if adding exogenous histidine to the medium could increase expression of his-tagged protein. It would be reasonable to hypothesize that methanogens may produce limiting quantities histidine to synthesize large quantities of a his-tagged protein. Histidine supplementation, assuming it could be transported into the cell and used to charge histinyl tRNAs, may alleviate this limitation and result in higher expression levels. However, addition of histidine had no measurable effect on protein expression. Finally, we verified that the UidA protein expressed from pNB730 was properly folded and active. As expected, cell extract from strains NB231

(*att*:pSK1, expresses untagged protein) and NB232 (*att*:pSK2, expresses dual-tagged protein) had detectable β-glucuronidase activity, whereas extract from the parent strain had no detectable β-glucuronidase activity (Figure 4). We noted that activity of untagged β-glucuronidase (pSK1) is higher than the dual-tagged β-glucuronidase (pSK2), demonstrating that amino and/or carboxy-terminal peptide tags can affect enzyme function and may not reflect differences in translation efficiency from identical promoters.

3.5 Creation of plasmids for expression of protein with cleavable affinity tags

In some circumstances (i.e., if it interferes with enzyme activity, or for protein crystallography) it may be preferable to have the ability to cleave affinity tags from expressed protein. Therefore we designed two additional plasmids based on pNB730, which include thrombin cleavage sites (Figure 2). The thrombin recognition site (LVPRGS) was optimized for expression in *M. acetivorans* [73, 74]. Plasmid pNB735 has the thrombin site immediately downstream of the 5' strep-tag and histidine tag, before the NcoI site where a gene of interest can be cloned. The complementary plasmid, pNB737, was created to express proteins with a thrombin-cleavable amino-terminal his tag. The sequences of both plasmids, pNB735 (Figure S10) and pNB737 (Figure S11), have been verified by DNA sequencing.

4. Discussion

We have succeeded in creating a suite of easy-to-use plasmids for gene deletion and expression of affinity tagged protein in *Methanosarcina acetivorans*. We have also demonstrated the utility of these plasmids in deleting genes from the chromosome (MA4421), and in expressing active enzymes in vivo. Depending on the restriction sites used for insertion of the gene of interest, the expressed protein either contains Strep-Tag II and histidine affinity tags or is untagged. The small, high-copy plasmids are compatible with ligation-independent cloning methods such as GeneArt Seamless Cloning and Assembly Kits (Invitrogen). The ease of propagation in *E. coli*, and the ease of cloning make the pNB723 and pNB730 family plasmids compatible with modern synthetic biology experiments. Though not demonstrated in this work,, plasmid pNB730 can be used for a wide array of experiments including mutant complementation, purification of proteins to study post-translational modification, and metabolic engineering applications in addition to expression of foreign proteins in the cell [75–77].

pNB735 and pNB737 plasmids will also make it easier to express protein in *Methanosarcina* for purification and crystallography purposes. To advance methanogen structure/function studies, we anticipate plasmid tools designed specifically for protein purification in methanogens, such as pNB735 and pNB737, may make it possible to obtain large quantities of pure, correctly folded protein from the native organism. Purification of protein from the native host may enable correct protein folding and population of the active site with the physiological cofactor. After purification, affinity tags can be removed by digestion with

thrombin protease. The pNEB193-derived plasmids we created add to the expanding repertoire of genetic and protein expression tools in *M. acetivorans* and other *Methanosarcina* species [5, 58, 59, 78, 79].

The multiple cloning site we designed for pNB730 and derivative plasmids contained a UAG stop codon to terminate translation of the carboxy-terminal his-strep affinity tag. In *Methanosarcina*, UAG can either be translated as a pyrrolysine residue, or will be recognized as a termination signal, depending on whether a PYLIS element is encoded in the 3' untranslated region of the RNA. When a PYLIS element is absent, approximately 70% of the translated polypeptides will stop at the UAG, while 30% of the time pyrrolysine will be incorporated into the growing protein chain, and translation continues until a second UAA or UGA stop codon is encountered. In the pNB730 multiple cloning site, the next in-frame stop codon is 336 bp downstream. If pyrrolysine had been incorporated in the *uidA* translation product, we would expect to detect two bands, one at 71 kDa, and the pyrrolysine read-through product at 83 kDa. In anti-strep immunoblots we only detected a single band at 71 kDa, indicating that translation was terminated at the first UAG codon. Kryzcki and coworkers noted that in highly expressed monomethylamine methyltransferase genes, the +1 and +2 nucleotides after the pyrrolysine-coding UAG codon are often GG [80]. Others have observed that the efficiency of pyl incorporation at UAG codons in heterologous systems can vary with the gene context [81, 82]. This contextual dependence on translational termination has been described in eukarya [83]. Our data suggests that the +1 and +2 nucleotides after the stop codon, TT, may disfavor pyrrolysine incorporation and instead results in translation termination in methanogens.

5. Conclusions

Methanogenic archaea produce several unusual coenzymes and cofactors that are not synthesized by *E. coli*, thereby constraining the ability to use *E. coli* as a heterologous host for overexpression and purification of a subset of methanogen proteins. To address this limitation, we have created a suite of plasmids for gene deletion and protein overexpression in *Methanosarcina* species. The new plasmids are derived from the small, high-copy *E. coli* plasmid, pNEB193, and can be propagated in standard *E. coli* cloning strains. We have successfully used the new plasmids to overexpress a native or his-strep tagged β-glucuronidase and to delete the gene MA4421 from the chromosome. These plasmids complement the growing list of genetic tools available for studying methanogen biology, and will be especially useful for identifying post-translational modifications in methanogen proteins, and for expressing proteins with amino- or carboxy-terminal affinity tags that can be cleaved with thrombin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We have created a suite of user-friendly plasmids for methanogens.
- The new plasmids are now compatible with ligation-independent cloning.
- We validated plasmids for markerless gene deletion
- Plasmids were used to express a native and his-tagged reporter gene.

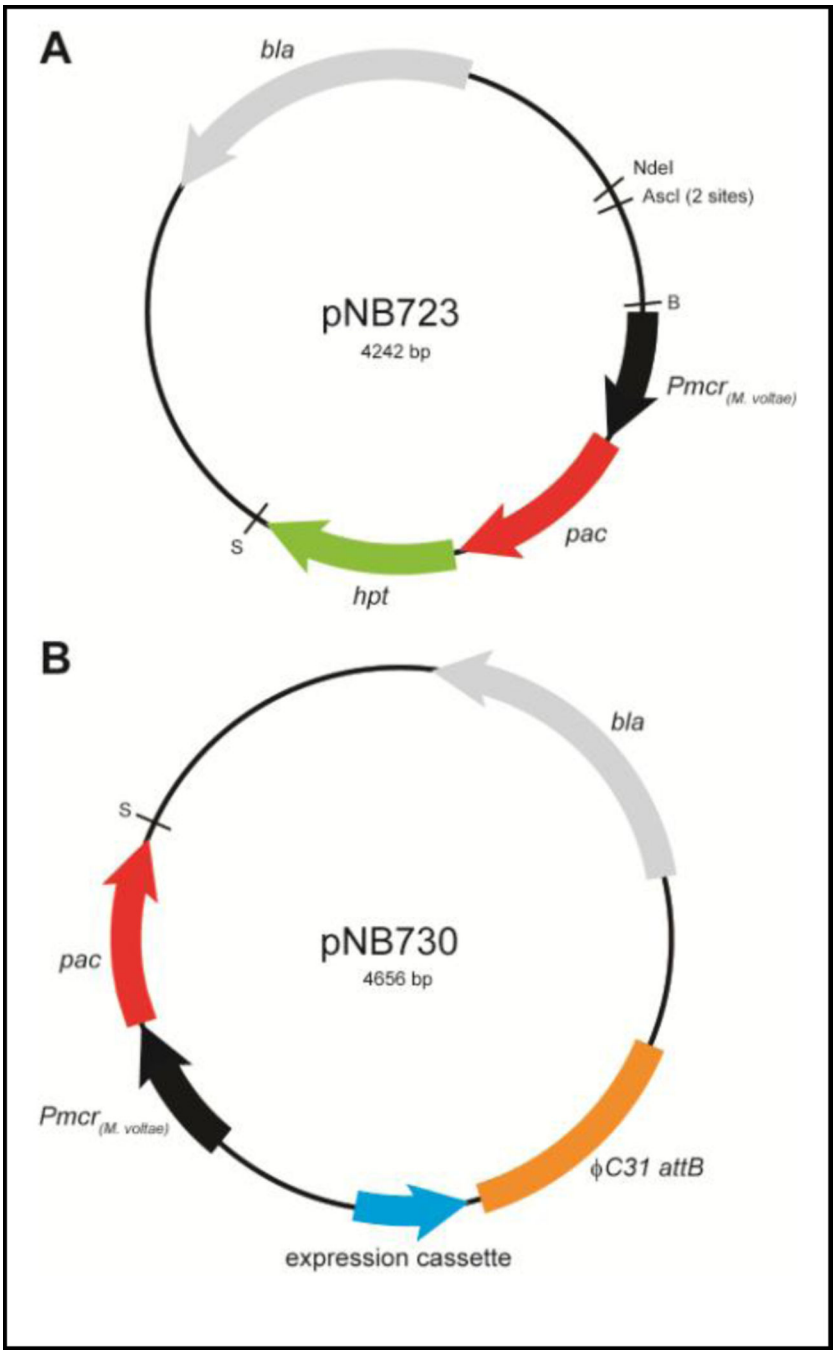

Figure 1.

pNB723 and pNB730 plasmid maps. Genes encoding puromycin acetyltransferase (*pac*, red) and hypoxanthine phosphoribosyltransferase (*hpt*, green) are codon-optimized for expression in *Methanosarcina*.

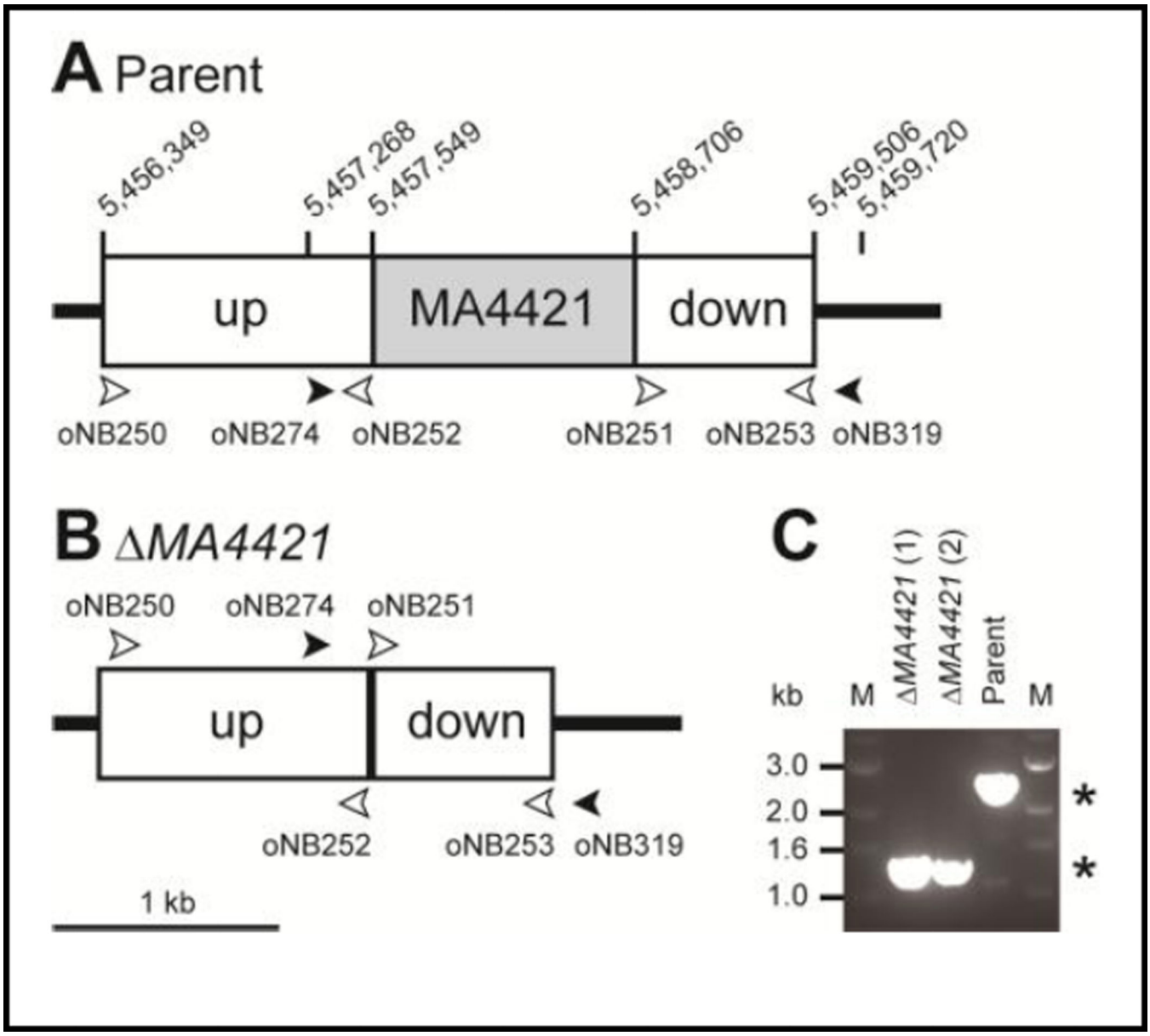

Figure 2.

Deletion of MA4421 from the chromosome using pALD1. Schematics of the *MA4421* genomic locus in the parental strain (A), and in the deletion mutant (B), are shown. (C) PCR results with oligos oNB274 and oNB319 showing deletion of *MA4421* in two isogenic isolates. Gray box= *MA4421* coding sequence. White boxes= DNA sequences upstream ("up") and downstream ("down") of the *MA4421* gene that were used to create plasmid pALD1. Open arrowheads= annealing site of PCR oligos used to construct pALD1. Solid arrowheads= annealing site of PCR oligos used to screen for deletion of *MA4421* on the chromosome. M= DNA size marker. Kb= kilobasepairs. The asterisks denote the expected amplicon sizes.

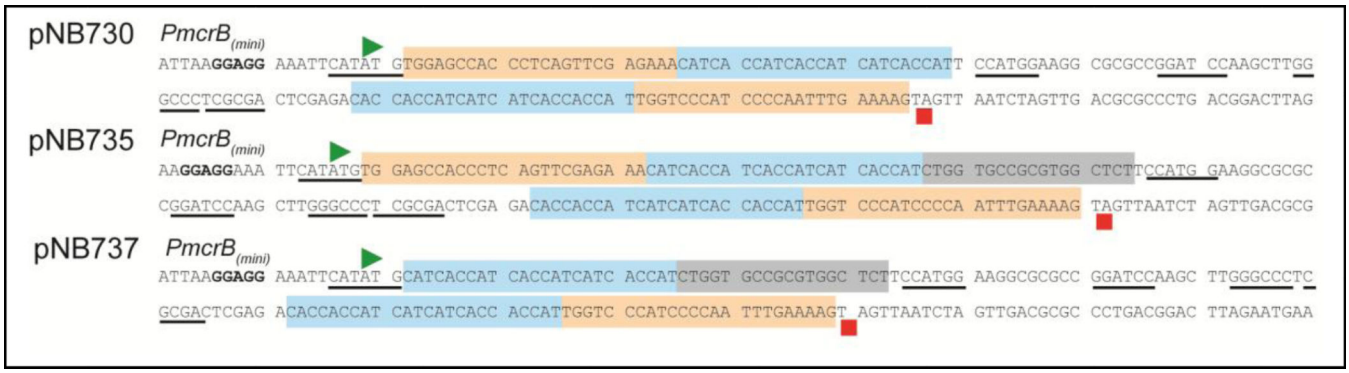

Figure 3.

Multiple cloning sites of pNEB193-derived plasmids used for protein expression in *Methanosarcina*. The ribosome binding site is in bold font. Unique restriction sites are underlined. Green triangle= translation start site. Red square= stop codon. Orange box= Strep-Tag II sequence. Blue box= histidine tag sequence. Gray box= thrombin recognition sequence.

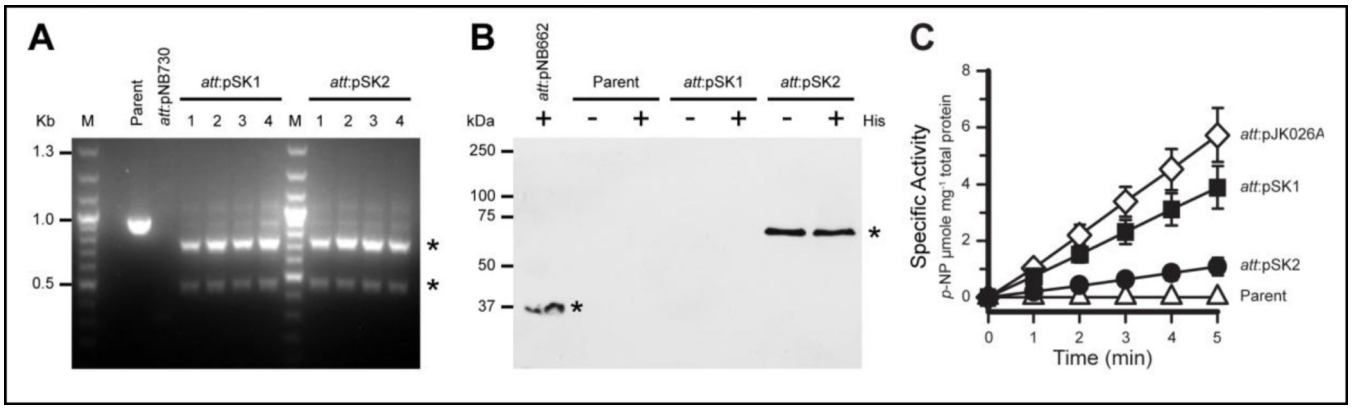

Figure 4.

Expression of tagged UidA protein in *M. acetivorans*. (A) Verification of integration of pSK1 and pSK2 on the chromosome. Genomic DNA from four isolates of each transformation were screened by PCR. M= marker. (B) Western blot of strep-his-UidA-his-strep protein expressed in *M. acetivorans*. Two micrograms of total protein from each strain were separated by denaturing PAGE. Western blots were probed with anti-strep antibodies. His= cultures were supplemented with 0.1 mM histidine. (C) Triplicate cultures of each strain were assayed in triplicate (N=9). Specific activity reported in nmoles min^{-1} mg^{-1} lysate.

Table 1

Strains used in this study

NB#	Genotype	Purpose	Reference
E. coli strains			
NB3	*5α F′ lacIq*	parent	New England Biolabs
NB4	*10β*	parent	New England Biolabs
NB100	*10β*/pNB721	Promotorless *pac* (*opt*) for conditional essentiality test of promoters in *M. acetivorans*	This study
NB101	*10β*/pNB722	*pac* (*opt*) vector for homologous recombination repair of mutants, gene deletion by homologous recombination (marked) in *M. acetivorans*	This study
NB104	*5α F′ lacIq*/pNB723	Gene deletion by homologous recombination (markerless) in *M. acetivorans*	This study
NB128	*5α F′ lacIq*/pNB724	*pac* (*opt*) vector for homologous recombination repair of mutants, gene deletion by homologous recombination (marked) (unique *SpeI* restriction site) in *M. acetivorans*	This study
NB131	*5α F′ lacIq*/pNB727	operon insertion into φC31 *attP* site on the chromosome in *M. acetivorans*	This study
NB133	*5α F′ lacIq*/pNB729	Expression of native or tagged protein in *M. acetivorans*	This study
NB134	*5α F′ lacIq*/pNB730	Expression of native or tagged protein (unique *BamHI* restriction site) in *M. acetivorans*	This study
NB161	*5α F′ lacIq*/pALD1	*MA4421* deletion by homologous recombination (markerless) in *M. acetivorans*	This study
NB224	*5α F′ lacIq*/pSK1	Expresses native UidA in *M. acetivorans*	This study
NB225	*5α F′ lacIq*/pSK2	Expresses strep-his-UidA-his-strep protein in *M. acetivorans*	This study
NB238	*10β* / pNB735	Expresses native or tagged protein in *M. acetivorans*. Amino-terminal strep-his tag is cleavable with thrombin protease.	This study
NB239	*10β*/ pNB737	Expresses native or tagged protein in *M. acetivorans*. Amino-terminal his tag is cleavable with thrombin protease.	This study
***M. acetivorans* strains**			
NB34	*Δhpt*::φC31 *int, attP*	parent	[59]
NB218	*Δhpt*::φC31 *int, attP, ΔMA4421*	*ΔMA4421* mutant	This study
NB231	*Δhpt*::φC31 *int, att*:pSK1	Expresses native UidA protein	This study
NB232	*Δhpt*::φC31 *int, att*:pSK2	Expresses strep-his-UidA-his-strep tagged protein	This study

Table 2

Sequences of DNA synthesized in this study

Purpose	Name	Sequence (5'-3')	Reference
Amplify *pac* (*opt*) gene from pMS86	oNB115	TCTAGAGTGATTCTCATGACCGAATATAAAC	This study
	oNB116	GCATGCACTAGTTCATGCTCCAGGTTTCCTG	This study
Amplify *PmcrB*$_{(M.\ voltae)}$ from pMP44	oNB128	ACTAGTCGGTTTGCGTATTGGCG	This study
	oNB129	ACTAGTTCCTATTTTTTTGATATATACATCATAACA	This study
Amplify *hpt* (*opt*) from pMS66	oNB106	TCTAGATCACTGATTTCCAAAAACATCTTTAATCTCAACTCC	This study
	oNB127	TCTAGACATGGTTGAAAGGCTTAAAGATTCCC	This study
Removes *SpeI* restriction site from pNB722	oNB151	GCATGCAAGCTTGGCGTAATCATG	This study
	oNB152	GCATGCTCATGCTCCAGGTTTCC	This study
Amplify φC31 *attB* from pJK026A	oNB117	TCTAGAATGAATCAACAACTCTCCTGGCGCA	This study
	oNB118	TCTAGACGCTGGCGATTCAGGTTCATCATG	This study
Amplify entire pNB724	oNB110	ACTAGTCTTGTCTGCTCCCGGCATCCG	This study
	oNB111	ACTAGTCCCGTCAGGGCGCGTCA	This study
Amplify entire pNB727	oNB130	ACTAGTGGTGTGAAATACCGCACAGATGCGTAA	This study
	oNB131	ACTAGTTGACGCGCCCTGACGGG	This study
Amplify expression cassette from pNB716 (inserts in pIDTSmart-Amp)	oNB183, M13 forward - 20	GTGTAAAACGACGGCCAGTTTATCTAGTCA	Integrated DNA Technologies (IDT)
	oNB184, M13 reverse -27	CCTCAGGAAACAGCTATGACATCAAGCT	Integrated DNA Technologies (IDT)
Removes second *BamHI* site from pNB729	oNB185	GGGGGCGCGCCGGATCTTAATTAAGTCTAG	This study
	oNB186	CTAGACTTAATTAAGATCCGGCGCGCCCCC	This study
Creation of *MA4421* deletion fusion fragment	oNB250	AAAAAAAAAAAAGGCGCGCCCTGGATTTTTTACAGATTCTAATGATTCCAGG	This study
	oNB251	GCTCTGCATATATCTTGGATCTTATACCCCATGCTGAACTACAGAACGTT	This study
	oNB252	AACGTTCTGTAGTTCAGCATGGGGTATAAGATCCAAGATATATGCAGAGC	This study
	oNB253	AAAAAAAAAAAAGGCGCGCCCCTGCCCCTCACATAATCGTGC	This study
	oNB311	GCCCTGGTTTGGTTCCCGGTTTACCAGAGAATGGAGGTATAAGATCCAAGAT	This study
	oNB312	ATCTTGGATCTTATACCTCCATTCTCTGGTAAACCGGGAACCAAACCAGGGC	This study
Amplifies *MA4421* chromosomal region	oNB274	GACCTTCTGGTGGATTGTTG	This study
	oNB318	GCAAAGCTTGTATACAGGGCAG	This study
	oNB319	CTCGGAAGCATGGTCTATCC	This study
Amplifies *uidA* from pJK026A	oNB369	CATATGTTACGTCCTGTAGAAACCCCAACCCG	This study
	oNB370	CCATGGTACGTCCTGTAGAAACCCCAACCCG	This study
	oNB371	GGATCCTCATTGTTTGCCTCCCTGCTGCGG	This study
	oNB372	GGATCCTTTTGTTTGCCTCCCTGCTGCGGTT	This study
pNB723 insert sequencing	oNB301	GGCTGGCTTAACTATGCGGCATC	This study
	oNB302	GCACCGTGGGTTTATATTCGGTCATGAGAATC	This study

Purpose	Name	Sequence (5'-3')	Reference
pNB730 MCS insert sequencing	oNB303	CGTCAGGGCGCGTCATTAACTACT	This study
att integration of pNB730 at the *hpt* locus	φC31 screenall#1	GAAGCTTCCCCTTGACCAAT	[59]
	φC31 screen-C2A#1	TTGATTCGGATACCCTGAGC	[59]
	φC31 screen- pJK200#1	GCAAAGAAAAGCCAGTATGGA	[59]
	oNB317	GATGAGTGGCAGGGCGGGGCGTAAT	This study
Thrombin-cleavable tagged protein	Strep-His-Thrombin	ATTAAGGAGGAAATTCATATG TGGAGCCACCCTCAGTTCGAGA AACA TCACCATCACCATCATCA CCATCTGGTGCCGCGTGGCTCT TCCATGGAAGGCGCGCCGGA TC CAAGCTTGGGCCCTCG	This study
	His-Thrombin	ATTAAGGAGGAAATTCATATGC ATCACCATCACCATCATCACCA TCT GGTGCCGCGTGGCTCTTCC ATGGAAGGCGCGCCGGATCCA AGCTTGGGCCCTCG	This study

Table 3

Plasmids used in this study

Name	Features	Purpose	Reference
pNEB193	*pUC19 ori*, *bla*$^+$, *lacZ*$^+$	Parent vector	New England Biolabs
pMP44	*oriR6K*, *bla*$^+$, $P_{mcr\ (M.\ barkeri)}$ *hpt*, $P_{mcr\ (M.\ voltae)}$ *pac*	Gene deletion by homologous recombination (markerless)	[58]
pJK026A	*oriV*, *repE*, *sopABC*, *cat*, φC31 *attB*, $P_{mcr\ (M.\ voltae)}$ *pac-hpt*, *PmcrB uidA*	Gene insertion into φC31 *attP* site on the chromosome	[59]
pMS86	*pac* (*opt*)	synthesized	This study
pMS66	*hpt* (*opt*)	synthesized	This study
pNB716	*strep-his MCS his-strep* expression cassette	synthesized	This study
pNB721	*pUC19 ori*, *bla*$^+$, *lacZ*$^+$, *pac* (*opt*)	Promotorless *pac* (*opt*) for conditional essentiality test of promoters in *M. acetivorans*	This study
pNB722	*pUC19 ori*, *bla*$^+$, *lacZ*$^+$, $PmcrB_{(M.\ voltae)}$ *pac* (*opt*)	*pac* (*opt*) vector for homologous recombination repair of mutants, KO by homologous recombination (marked) in *M. acetivorans*	This study
pNB723	*pUC19 ori*, *bla*$^+$, *lacZ*$^+$, $P_{mcr\ (M.\ voltae)}$ *pac- hpt*	Gene deletion by homologous recombination (markerless) in *M. acetivorans*	This study
pNB724	*pUC19 ori*, *bla*$^+$, *lacZ*$^+$, $PmcrB_{(M.\ voltae)}$ *pac* (*opt*)	*pac* (*opt*) vector for homologous recombination repair of mutants, gene deletion by homologous recombination (marked) (unique SpeI restriction site) in *M. acetivorans*	This study
pNB727	*pUC19 ori*, *bla*$^+$, *lacZ*$^+$, φC31 *attB*, $PmcrB_{(M.\ voltae)}$ *pac* (*opt*)	operon insertion into φC31 *attP* site on the chromosome	This study
pNB729	*pUC19 ori*, *bla*$^+$, *lacZ*$^+$, φC31 *attB*, $PmcrB_{(M.\ voltae)}$ *pac* (*opt*), *strep-his MCS his-strep* expression cassette	Expression of native or tagged protein in *M. acetivorans*	This study
pNB730	*pUC19 ori*, *bla*$^+$, *lacZ*$^+$, φC31 *attB*, $PmcrB_{(M.\ voltae)}$ *pac* (*opt*), *strep-his MCS his-strep* expression cassette	Expression of native or tagged protein (unique BamHI restriction site) in *M. acetivorans*	This study
pALD1	*pUC19 ori*, *bla*$^+$, *lacZ*$^+$, $PmcrB_{(M.\ voltae)}$ *pac- hpt*, *ΔMA4421* deletion fusion	*MA4421* deletion by homologous recombination (markerless) in *M. acetivorans*	This study
pSK1	*pUC19 ori*, *bla*$^+$, *lacZ*$^+$, φC31 *attB*, $PmcrB_{(M.\ voltae)}$ *pac* (*opt*), *uidA*	Expresses native UidA in *M. acetivorans*	This study
pSK2	*pUC19 ori*, *bla*$^+$, *lacZ*$^+$, φC31 *attB*, $PmcrB_{(M.\ voltae)}$ *pac* (*opt*), *strep-his- uidA-his-strep*	Expresses strep-his-UidA-his-strep protein in *M. acetivorans*	This study
pNB735	*pUC19 ori*, *bla*$^+$, *lacZ*$^+$, φC31 *attB*, $PmcrB_{(M.\ voltae)}$ *pac* (*opt*), *strep-his* (*thrombin*) *MCS his-strep* expression cassette	Expression of native or tagged protein in *M. acetivorans*. Amino-terminal strep-his tag is cleavable with thrombin protease.	This study
pNB737	*pUC19 ori*, *bla*$^+$, *lacZ*$^+$, φC31 *attB*, $PmcrB_{(M.\ voltae)}$ *pac* (*opt*), *his* (*thrombin*) *MCS his-strep* expression cassette	Expression of native or tagged protein in *M. acetivorans*. Amino-terminal his tag is cleavable with thrombin protease.	This study