Plasmid 84–85 (2016) 27–35

Contents lists available at ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

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

Mitchell T. Shea, Mary E. Walter, Nikolas Duszenko, Anne-Lise Ducluzeau, Jared Aldridge, Shannon K. King, Nicole R. Buan *

Redox Biology Center, Department of Biochemistry, University of Nebraska-Lincoln, Lincoln, NE, United States

ARTICLE INFO

Article history:
Received 28 August 2015
Received in revised form 10 February 2016
Accepted 10 February 2016
Available online 11 February 2016

Keywords:
Archaea
Protein expression
Methanogen
Methanosarcina

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 *Escherichia 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, *M. acetivorans*.

1. Introduction

Genetic methods for *Methanosarcina* species are well developed, and making mutations on the chromosome is a routine procedure in several laboratories (Ehlers et al., 2011; Buan et al., 2011; Whitman et al., 1997; Bertani and Baresi, 1987; Mondorf et al., 2012). 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 (Thauer, 1998). They employ unique enzymes and cofactors to activate carbon for reduction, and simultaneously generate a transmembrane ion gradient that is used for ATP synthesis (Deppenmeier, 2004; Kulkarni et al., 2009). Unusual cofactors used in methanogens include coenzyme M, coenzyme B, methanopterins, methanophenazine, dimethylbenzimidazolyl cobamide, and deazaflavin F_{420} (Bobik and Wolfe, 1988; Ankel-Fuchs et al., 1987; Leigh et al., 1985; Gunsalus and Wolfe, 1977; Wood and Wolfe, 1966; Mayr et al., 2008; Hinderberger et al., 2006; Kruger et al., 2003; Fischer and Thauer, 1990; Ankel-Fuchs and Thauer, 1986; Eirich et al., 1978; Keltjens and Vogels, 1988; van Beelen et al., 1984; Keltjens et al., 1983a; Keltjens et al., 1983b; Abken et al., 1998; Pol et al., 1982). 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 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 (Harms and Thauer, 1996; Kengen et al., 1992; Krautler et al., 1987). The methanogen methanol:corrinoid methyltransferase, MtaB, and the corrinoid protein, MtaC, from *Methanosarcina barkeri*, have been purified from *Escherichia coli* and biochemically characterized (Sauer and Thauer, 1999; Sauer and Thauer, 1998; Sauer and Thauer, 1997). 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 artifacts 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* (Hagemeier et al., 2006).

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* (Eirich et al., 1978; Klein and Thauer, 1995; Kunow et al., 1993; Jaenchen et al., 1984; Anon., 1980). To our knowledge, no predicted flavin-binding proteins

* Corresponding author.
E-mail address: nbuan@unl.edu (N.R. Buan).

http://dx.doi.org/10.1016/j.plasmid.2016.02.003
0147-619X/

from methanogens have 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* (Fielding et al., 2013; Wang et al., 2014; Lessner and Ferry, 2007; Korbas et al., 2006; Hedderich et al., 2005; Lyon et al., 2004; Takahashi and Tokumoto, 2002; Zhao et al., 2001; Bertram et al., 1994). 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-} (Kulzer et al., 1998).

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 (Grayling et al., 1995; Ehlers et al., 2002; Richter et al., 2012; Ehlers et al., 2005). 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 (Torarinsson et al., 2005; Karlin et al., 2002; Bell and Jackson, 1998; Koonin et al., 1997). 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 (Makrides, 1996; Saxena and Walker, 1992; Makoff et al., 1989). The yield of heterologously expressed methanogen protein can be increased by codon optimizing the nucleotide sequence for *E. coli* (Makrides, 1996; Makoff et al., 1989). 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 (Pritchett et al., 2004; Guss et al., 2008). pMP44 is useful for markerless deletion of genes using homologous recombination (Pritchett et al., 2004). However, pMP44 replicates in the *E. coli* host at a relatively low-copy number and must be propagated in a *pir*$^+$ strain (Filutowicz et al., 1986; Stalker et al., 1983). Plasmid pJK026A and its derivatives can be used for inserting DNA at a ϕC31 phage *att* site which has been added to the chromosome (Guss et al., 2008). It is useful for expressing protein in *Methanosarcina*, or for studying transcription and translational fusions (Buan et al., 2011). pJK026A family plasmids are 11.7 kb, and must be purified from a *trfA*$^+$ *E. coli* strain (Thomas et al., 1985). 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 *Methanosarcina acetivorans* chromosome and be used for in vivo protein expression and purification via Strep-Tag II and histidine affinity tags (Ko et al., 1994; Voss and Skerra, 1997; Schmidt and Skerra, 1994).

2. Materials and methods

2.1. Growth of cultures

E. coli was grown in Lysis Broth (LB) at 37 °C with shaking (Uetake et al., 1958). *M. acetivorans* strains were grown at 35 °C in HS medium as described (Metcalf et al., 1996). 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

Table 1
Strains used in this study.

NB#	Genotype	Purpose	Reference
E. coli strains			
NB3	*5α F′ laclq*	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′ laclq*/pNB723	Gene deletion by homologous recombination (markerless) in *M. acetivorans*	This study
NB128	*5α F′ laclq*/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′ laclq*/pNB727	Operon insertion into ϕC31 *attP* site on the chromosome in *M. acetivorans*	This study
NB133	*5α F′ laclq*/pNB729	Expression of native or tagged protein in *M. acetivorans*	This study
NB134	*5α F′ laclq*/pNB730	Expression of native or tagged protein (unique *BamHI* restriction site) in *M. acetivorans*	This study
NB161	*5α F′ laclq*/pALD1	*MA4421* deletion by homologous recombination (markerless) in *M. acetivorans*	This study
NB224	*5α F′ laclq*/pSK1	Expresses native UidA in *M. acetivorans*	This study
NB225	*5α F′ laclq*/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	Guss et al. (2008)
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