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Full Length Research Paper

Using polymerase chain reaction (PCR) melting profile and protein fingerprint profile analyses to identify *Methanothermobacter marburgensis* DX01

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A thermophilic new strain of *Methanothermobacter marburgensis* DX01, with a different morphology from *M. thermautotrophicus* delta H, was isolated from a hot spring in China. To provide further molecular evidence to identify the methanogen *M. marburgensis* DX01, polymerase chain reaction melting profile (PCR MP) technique and protein fingerprint profiling were performed. Both DNA and protein fingerprint profiles showed significant differences between *M. marburgensis* DX01 and *M. thermautotrophicus* delta H. In addition, five interesting DNA fragments obtained from PCR MP of the DX01 strain were excised and sequenced. We found that all five DNA fragments were unique to *M. marburgensis* DX01. Expression of induced proteins in *M. marburgensis* DX01 was analyzed by protein fingerprint profiling and a protein that was more highly expressed at 70°C was excised and identified by N-terminal amino acid sequencing. We found that the up-regulated protein was Methyl-coenzyme M reductase I, which was 97.6% amino acid sequence identity with that of *M. thermautotrophicus* delta H. These studies may provide clues for a better understanding of the new isolate of thermophilic *M. marburgensis*.

Key words: *Methanothermobacter,* polymerase chain reaction (PCR) melting profile, protein fingerprint profile analyses.

INTRODUCTION

There are a large number of extremely thermophilic organisms in the archaeal domain (de Poorter et al., 2007; Schill et al., 1996). The thermophilic species of *Methanothermobacter* are able to produce their energy from the oxidation of H_2 and reduction of CO_2 to methane (Valentine, 2007). Growth of *Methanothermobacter* is characterized by a requirement for gaseous energy and carbon sources and low biomass. In addition, *Methanothermobacter* is thermophilic and strictly anaerobic (Farhoud et al., 2005; Schill et al., 1996). Currently, over ten *Methanothermobacter* strains have been found in a number of environments, including anaerobic digesters, digested sludge and hot springs. Although these strains were assigned to a single species, there are still certain differences between the strains. With the development of new taxonomy techniques, various taxonomic subdivisions of *Methanothermobacter* into separate species have been proposed. In 2001, *Methanothermobacter* was redefined as *Methanothermobacter defluvii*, *Methanothermobacter marburgensis, Methanothermobacter thermautotrophicus, Methanothermobacter thermoflexus, Methanothermobacter thermophilus* and *Methanothermobacter wolfeii*, as cited in the second edition of the Bergey's Manual of Systematic Bacteriology and published in the validation list no. 85.

Over the past 20 years, a large number of DNA-based techniques have been introduced into the field of microbial characterization and taxonomy. These include

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restriction endonuclease analysis, molecular hybridization, and PCR amplification; most of these techniques use PCR for detection of fragments (Dnyaneshwar et al., 2006; Frias-Lopez et al., 2008; Krawczyk et al., 2006; Lopez-Ribot et al., 2000; Pandya et al., 2009). These genomic fingerprinting methods were developed to detect DNA sequence polymorphisms by using general principles such as restriction fragment length polymorphisms (RFLPs) (Botstein et al., 1980), random amplification of polymorphic DNA (RAPD) (Williams et al., 1990), or repetitive element PCR (rep-PCR) (Georghiou et al., 1994).

Nowadays, physiological characterization, phylogenetic analysis of the 16S rRNA, DNA-DNA hybridization, analysis of the GC percentage, antigenic fingerprinting, and plasmid and phage typing have all been used for type strains of *Methanothermobacter* (Wasserfallen et al., 2000). The performance and convenience of the PCR melting profile (PCR MP) technique, which is based on using low denaturation temperatures during ligation mediated PCR (LM PCR) of bacterial DNA, demonstrates that PCR MP is a rapid method that offers good discriminatory power (Krawczyk et al., 2009; Krawczyk et al., 2007; Krawczyk et al., 2006; Masny and Pluciennicza k, 2003; Mueller et al., 2001; Ngoc et al., 2008).

A new thermophilic archaea *M. marburgensis* DX01 was isolated from a hot spring in China (Ding et al., 2010). The isolate showed some distinct phenotypic characteristics in pellet color and morphology. Previously, *M. marburgensis* and *M. thermautotrophicus* were thought to belong to the same species: *Methanobacterium thermoautotrophicum*. Strain delta H was a type strain. The two species exhibited extremely similar physiological characteristics. However, DNA-DNA hybridization studies revealed they lacked similarity in sequence (Wasserfallen et al., 2000). The differences between the two species in genomic sequence and protein expression were still unknown. Additionally, it was not known whether the new DX10 strain possessed unique genes. In the present study, to provide further molecular evidence of the difference between the strains, PCR MP and protein fingerprint profiles were used to create a more complete picture of the variation in genomes and protein expression to fingerprint a novel methanogen strain DX01.

MATERIALS AND METHODS

Culturing

Methanothermobacter strains were grown in anaerobic medium using the Hungate technique (Balch et al., 1979). The culture medium contained (L^{-1}): $MgCl_2 \cdot 6H_2O$, 0.1 g; NaCl 0.6 g; K_2HPO_4, 1.5 g; NH_4Cl, 1.5 g; sodium cysteine 0.5 g; $NaHCO_3$, 1.0 g; sodium resazurin 0.001 g; trace element solution 10 ml ($MgSO_4 \cdot 7H_2O$ 3 g; $MnSO_4 \cdot 4H_2O$ 0.5 g; NaCl 10 g; $FeSO_4 \cdot 7H_2O$ 0.1 g; $CoCl2 \cdot 6H_2O$ 0.1 g; $CaCl_2 \cdot 2H_2O$ 0.1 g; $ZnSO_4 \cdot 7H_2O$ 0.1 g; $CuSO_4$ 0.01 g; $KAl(SO_4)_2 \cdot 12H_2O$ 0.01 g; H_3BO_3 0.01 g; $Na_2MoO_4 \cdot 2H_2O$ 0.01 g; $NiCl_2 \cdot 6H_2O$ 0.025 g; $NaSeO_3 \cdot 6\ H_2O$ 0.0003 g). After adjustment to pH 7.0, the medium was prepared and sterilized under a strictly anaerobic H_2 and CO_2 atmosphere (80:20).

Electron microscopy (EM) at different growth temperature

Strains were grown to late exponential phase at different incubation temperatures (50, 60, 65 and 70°C). The electronic graph was performed using electron microscopy (EM).

DNA extraction

Chromosomal DNA was obtained using previously described methods (Jarrell et al., 1992). Total DNA was quantified on a Genova UV/visible spectrophotometer at 260 nm.

Ligation mediated PCR performed at low denaturation temperatures (PCR melting profiles)

The PCR melting profile (PCR MP) was performed according to previously described methods (Masny and Pluciennicza k, 2003). Briefly, genomic DNA was digested with HindIII. Digested DNA was ligated with two oligonucleotides forming an adaptor: POWIE 5'-CTCACTCTCACCAACGTCGAC-3' and HINLIG 5'-AGCTGTCGACGTTGG-3'. The ligation products were used in the next round of PCR as an amplification template with primer POWAGCTT 5'-CTCACTCTCACCAACGTCGACAGCTT-3'. PCRs were performed by incubating the samples for 2 min at 72°C to release unligated HINLIG oligonucleotides, followed by the addition of 1 µl of Taq DNA polymerase (2.5 U/µl). The reaction mixture was incubated for an additional 5 min at 72°C to fill in the single-stranded ends and create amplicons, after which the samples were initially denatured at (80 + x) °C for 1 min, followed by 21 cycles of denaturation at (80 + x) °C for 40 s, with annealing and elongation at 72°C for 2 min (where x is a digit between 0 and 8, and is constant for a single PCR reaction).

Comparison of PCR MPs of two strains

The products of PCR MPs were separated by 2% agarose gels. Electrophoresis was performed in 1·Tris–acetate–EDTA buffer at 50 V. Gels were stained by ethidium bromide. Interesting DNA fragments were recovered from the agarose gel using the Agarose Gel DNA Fragment Recovery Kit (TaKaRa, Dalian, China). Recovered PCR product was ligated into the pUCm-T vector (TaKaRa, Dalian, China) and transformed into *E. coli* DH5α cells. The cloning procedure was conducted according to the manufacturer's instructions. Sequencing reactions were run on an Applied Biosystems 3730 sequencer (Invitrogen, Shanhai, China).

N-terminal protein sequencing

Purified proteins from the fractions were first separated by SDS-PAGE and then transferred via electroblot onto PVDF membranes. Protein bands on the PVDF membrane were visualized by staining with G-250 Coomassie stain solution, after which protein bands of interest with the correct molecular weight were cleaved and the N-terminal amino acid sequence was determined on the Procise-Protein Sequencing System (Applied Biosystems) using the manufacturer's pulse-liquid Edman degradation chemistry cycles.

Cloning and nucleotide sequencing

Degenerate primers (5'-ATG ATA TGG CAC AAT ACT A-3' and 5'-

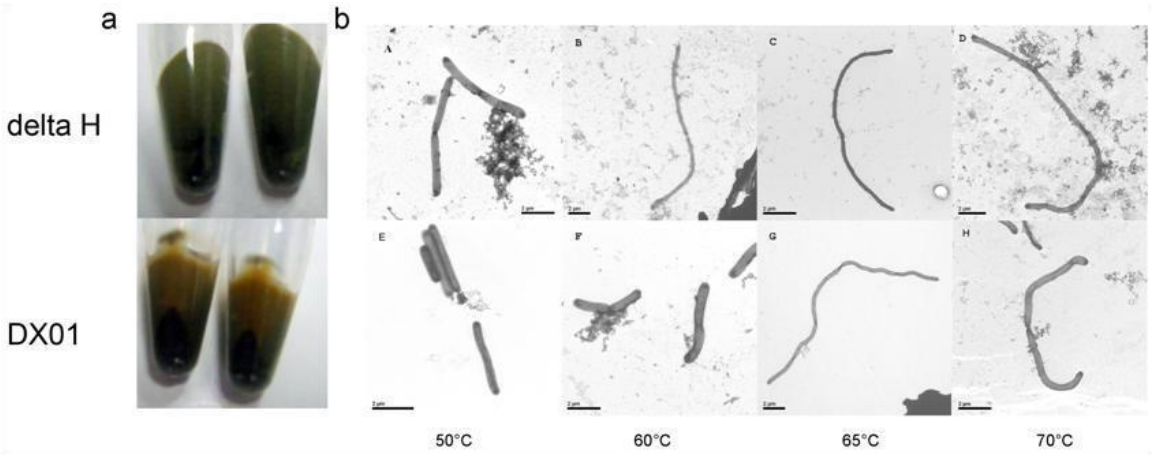

Figure 1. Morphology of *M. marburgensis* DX01. (a) Color of the cell pellet. (b) Electron microscopy of cells grown at 50°C (A, E), 60°C (B, F), 65°C (C, G), 70°C (D, H). A-D: *M. thermautotrophicus* delta H^T; E-H: *M. marburgensis* DX01. Bar indicates 2 µm.

TAG ATT ATG GCT GAC AAA T-3') were designed based on the N-terminal amino acid sequences. The fragment was amplified by PCR. The PCR product was ligated into the pUCm-T vector and sequenced. The sequence was deposited in GenBank (EU807736) and aligned to reference sequences.

RESULTS

The morphology of *M. marburgensis* DX01

It was interesting to find that the pellet of *M. marburgensis* DX01 was a brick red color that was very different from the *M. thermautotrophicus* delta H wild-type strain, which has a green and black pellet under the same culture conditions (Figure 1a). To further investigate the effect of temperature on the morphology of *M. marburgensis* DX01, the strain morphology at different temperatures was observed using electron microscopy. As shown in Figure 1b, the strain morphology was greatly influenced by the growth temperature. *M. marburgensis* DX01 grows as short rods at 50 and 60°C, while at 65 and 70°C, the strain grows as long, irregularly curved rods. Similar to *M. marburgensis* DX01, *M. thermautotrophicus* delta H appeared as short rods at 50°C and long irregularly curved rods at 65 and 70°C. However, *M. marburgensis* DX01 grew as short rods at 60°C, in contrast to *M. thermautotrophicus* delta H^T, which grew as long, irregularly curved rods at the same temperature.

Using PCR melting profiles to perform molecular typing of the *Methanothermobacter* isolate

To provide direct molecular evidence that *M.* the PCR MP *marburgensis* DX01 was a distinct methanogen species, technique for fingerprinting of *Methanothermobacter* was used. The obtained PCR MP fingerprinting patterns for representative *Methanothermobacter* strains are presented in Figure 2. More DNA fragments were amplified at higher Td values than at lower Td values, and each pattern consisted of approximately 2 to 25 fragments in the 100 to 1,200 bp size range (Figures 2A and 2B). In addition, PCR MP fingerprinting analysis demonstrated significant differences between *M. marburgensis* DX01 and *M. thermautotrophicus* delta H (Figures 2C and 2D). Therefore, the data presented here demonstrates that the genomes of the two *Methanothermobacter* strains possess substantial genomic heterogeneity.

To further confirm the diversity of DNA sequences, five different DNA fragments from *M. marburgensis* DX01 at T_d 83.1°C were excised and sequenced randomly (Figure 2D). The sequence of clone 1 was similar to several hypothetical proteins in different microorganisms as shown in Figure 3A. Alignment revealed that the sequence of clone 1 had 51.7, 35.8, and 29.8% identity at the protein level with *Thermotoga petrophila, Dictyoglomus thermophilum* and *Coprothermobacter proteolyticus*, respectively.

Clone 3 had an unclear sequence except for 36 bp that were identical with *Methanobacterium* phage psiM2 (data not shown). DNA sequence of clone 4 (235 bp), which spanned the promoter and the ORF, showed 90% similarity with translation initiation factor (eIF-5A) of *M. thermautotrophicus* delta H^T. The translated DNA of the ORF was exactly identical between the two species, and the variation only occurred at the third codon (Figure 3B). BLAST results showed no similarity between the strains for the sequences from clone 2 and 5. Taken together, these results indicated that the genome of strain DX01 is substantially different from that of *M. thermautotrophicus*

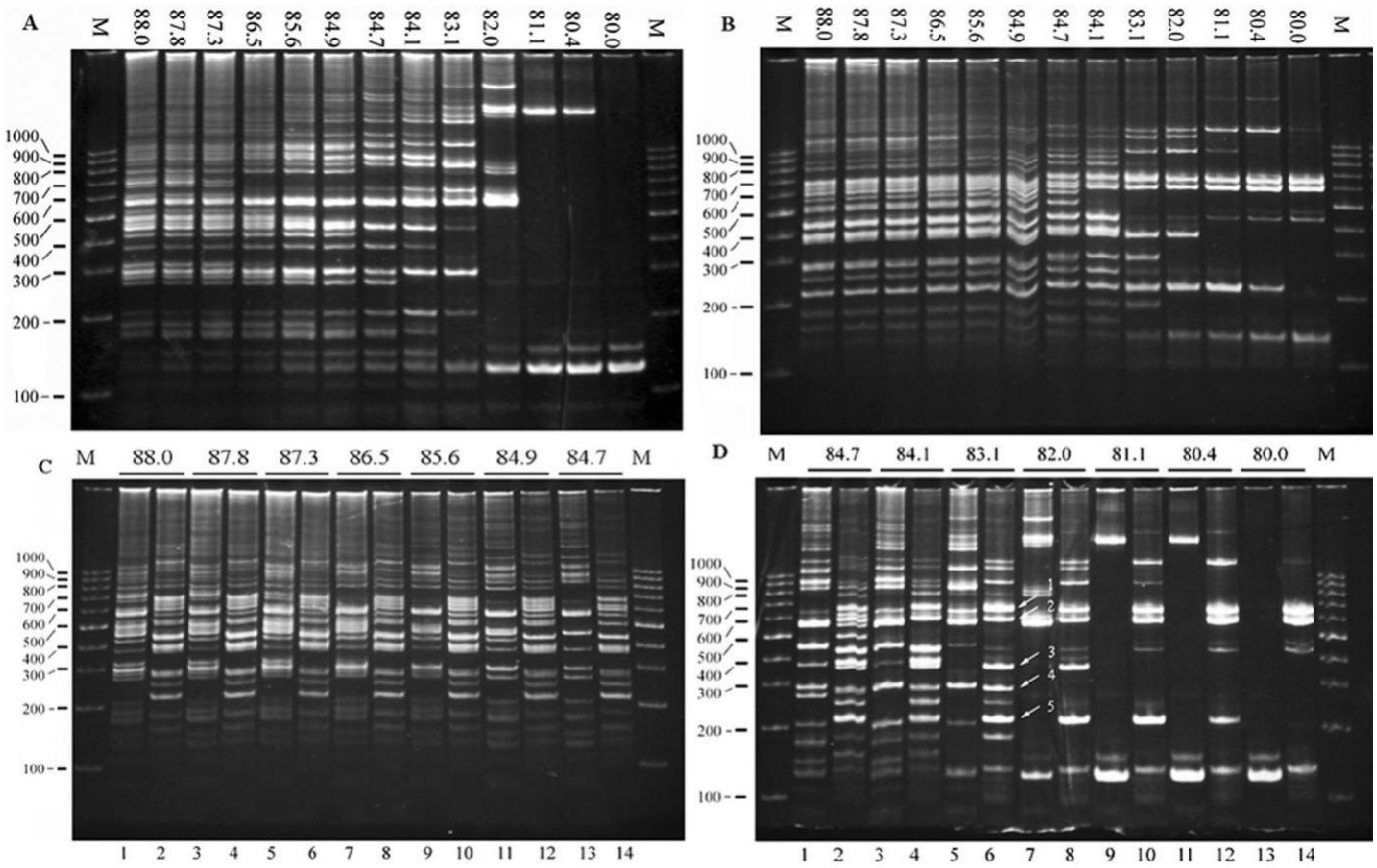

Figure 2. PCR melting profiles used in analyzing the molecular typing of *Methanothermobacter*. T_d gradients of 80-88°C were performed. Lane M: DNA molecular weight marker; fragment lengths in bp are indicated. (A) *M. thermautotrophicus* delta H^T; (B) *M. marburgensis* DX01; (C-D) Adjacent lanes with the same T_d are shown with an underline. Odd-numbered and even-numbered lanes were loaded with *M. thermautotrophicus* delta H^T and *M. marburgensis* DX01, respectively. Sequencing clones are marked.

delta H^T, and that the diverse DNA sequences of strain DX01 were unique for *M. marburgensis* DX01.

Using protein fingerprint profiles to examine proteins of *Methanothermobacter*

To better understand the response of *M. marburgensis* DX01 to different temperatures, different expressed proteins were identified by protein fingerprint profiles. Figure 4A represents the SDS-PAGE gel in which the protein expression levels are compared between cells incubated at different growth temperatures. The protein expression profiles of *M. thermautotrophicus* delta H were similar at all culture temperatures tested. In contrast, the protein expression profile of *M. marburgensis* DX01 at 70°C were observably different compared to protein expression profiles of cells grown at 50 and 60°C. These results provide direct molecular evidence demonstrating the existence of a significant difference between the two strains in protein expression.

Expression of induced proteins in *M. marburgensis* DX01 was analyzed with protein fingerprint profiles. A protein that appeared to have higher expression at 70°C was excised and identified by N-terminal amino acid sequencing (Figure 4A and B). Degenerate primers were designed based on the N-terminal amino acid sequences of the purified 30-kDa subunits, which corresponded to Methyl-coenzyme M reductase I. A fragment of approximately 750 bp was amplified by PCR. Sequencing this fragment indicated that there was 97.6% amino acid sequence identity of the Methyl-coenzyme M reductase I, gamma subunit gene between *M. thermautotrophicus* delta H^T and *M. marburgensis* DX01 (Figure 4C).

DISCUSSION

Methanothermobacter strains are members of the *Archaea* domain, and they have been isolated from patterns in response to different temperatures various environments (Kozono et al., 2003; Mochimaru et al., 2007). Unlike many other microorganisms, *Methanothermobacter* grows at particularly high extremes.

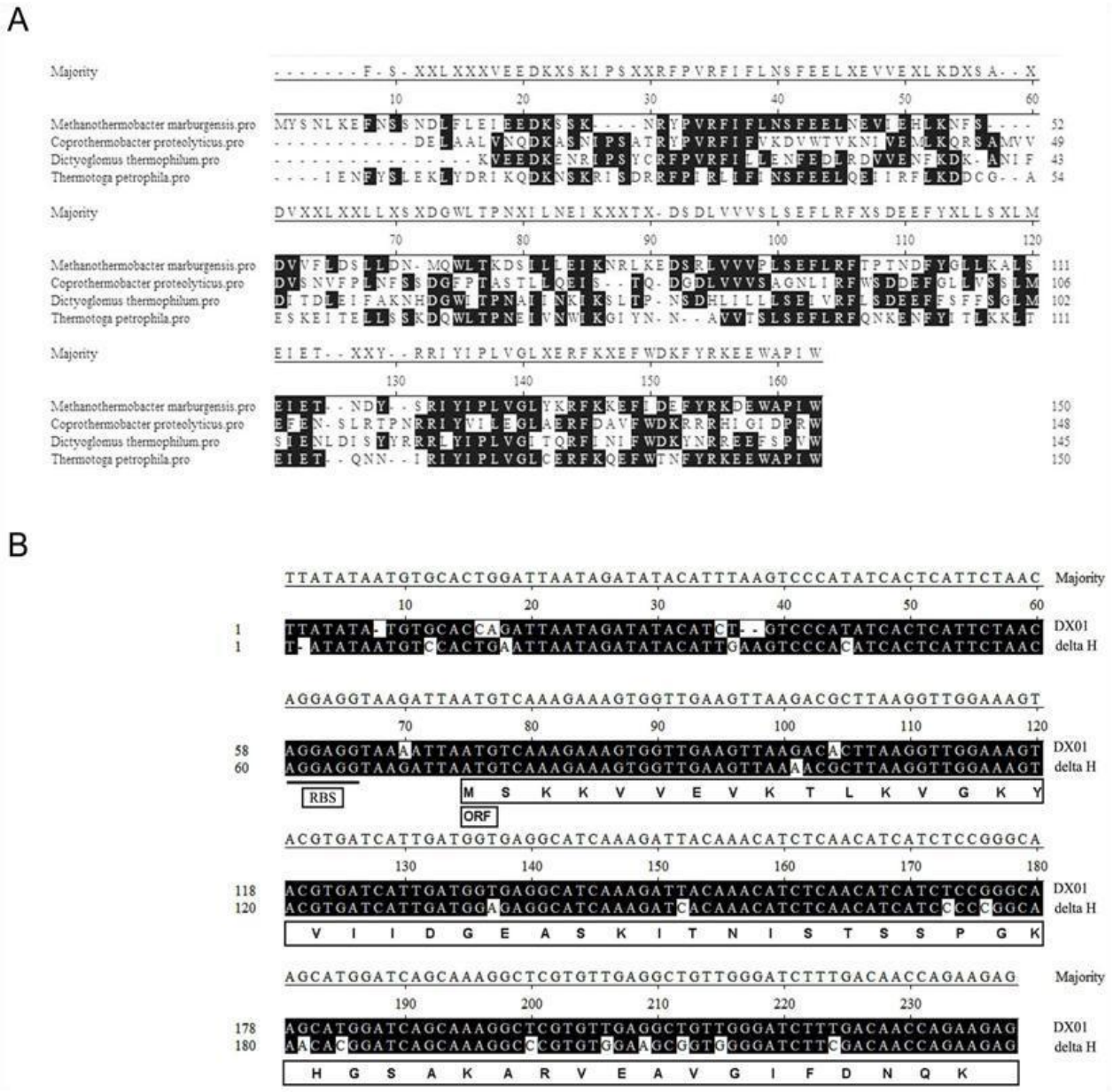

Figure 3. Alignment of the protein sequences. (A) Hypothetical proteins. (B) eIF-5A sequences. RBS: predicted ribosome binding site.

The biochemistry of methane formation in these organisms has been extensively studied. *Methanothermobacter* has been studied by different molecular techniques to analyse its essential genes. In addition, new members of the *Methanothermobacter* genus are continually isolated from various environment to extend the phylogenetic tree of this ancient family (Wasserfallen et al., 2000). However, multiple new techniques need to be used to analyze these archaea to identify new species. Here, we show the applicability of the PCR MP technique in identifying methanogenic archaea.

M. marburgensis DX01 was a new thermophilic methanogenic archaea isolated from a hot spring in China. The physiological characteristics of *M. marburgensis* DX01 had been studied previously (Ding et al., 2010). In this paper, morphological analysis showed that *M. marburgensis* DX01 demonstrated some distinct phenotypic characteristics, such as growth as short rods at 60°C and a brick red pellet, both of which are different from *M. thermautotrophicus* delta H (Figure 1). The special morphological characteristics were interesting and unique. To have a more accurate molecular understanding of the new strain of *M. marburgensis* DX01,

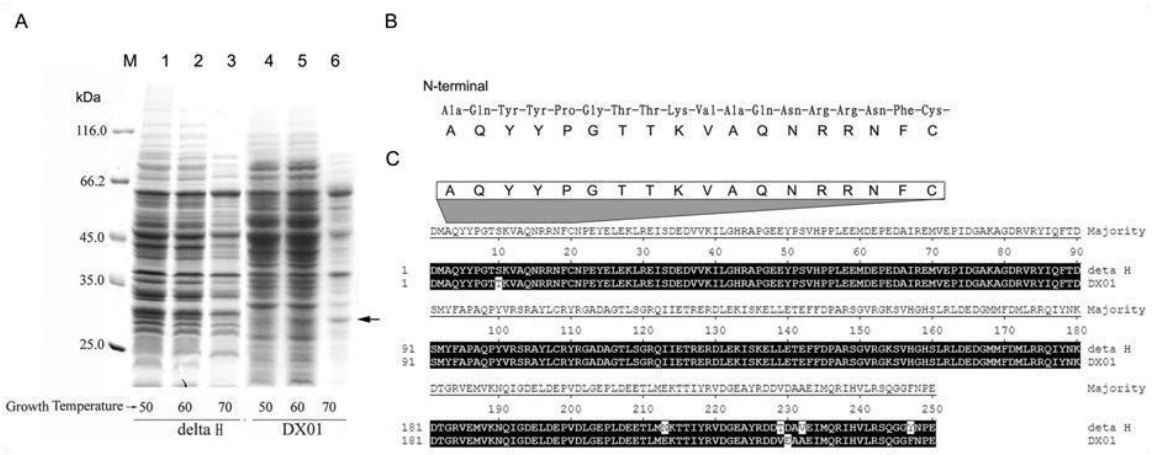

Figure 4. Identification of regulated proteins in response to temperature. A, Protein fingerprint profile analysis at different incubation temperatures. Lane M, protein molecular weight marker; fragment lengths in kDa are indicated. Lane 1-3, *M. thermautotrophicus* delta H^T; 4-6, *M. marburgensis* DX01; 1, 50°C; 2, 60°C; 3, 70°C; 4, 50°C; 5, 60°C; 6, 70°C. The protein extracted for sequencing is marked. B, N-terminal amino acid sequences of a protein with increased expression at 70°C. C, Alignment of the methyl-coenzyme M reductase I, gamma subunit protein sequences.

PCR melting profile technique and protein fingerprint profiles were used to characterize the genome and protein expression level differences.

The result of PCR melting profile analysis indicated that the genomes of these two methanogens possess substantial genomic heterogeneity (Figure 2). Five diverse DNA fragments from *M. marburgensis* DX01 were excised and sequenced randomly. BLAST results showed that no similarity between the two methanogens using the sequences of clone 1, 2 and 5. These results showed that there are unique DNA sequences in the *M. marburgensis* DX01 chromosomal DNA. In addition, the sequence of clone 1 was similar to several hypothetical proteins from *T. petrophila, D. thermophilum* and *C. proteolyticus* (Figure 3A). It was interesting that these microorganisms were all obligate anaerobes and extremely thermophilic. It was possible that this hypothetical protein is involved in responding to the stresses of extreme heat and anaerobic growth. Clone 3 had an unclear sequence except for 36 bp that were identical to *Methanothermobacter* phage psiM2. We hypothesize that *M. marburgensis* DX01 was infected by phage psiM2, and that fragment of phage psiM2 DNA were recombined into the *M. marburgensis* DX01 chromosomal DNA. The DNA sequence of clone 4, which spanned both the promoter and the ORF, showed 90% similarity with the translation initiation factor (eIF-5A) of *M. thermautotrophicus* delta H^T. We observed that the translated amino acids from this ORF were exactly identical to the *M. thermautotrophicus,* and that the variability in sequence only happened at the third codon (Figure 3B). This indicated that the DNA sequences are easy to mutate between the different strains, but the protein sequences are highly conserved. Additionally, we noticed that the promoter sequences were different between the strains.

Protein fingerprint profiles at different culture temperatures (50, 60 and 70°C) were performed and the results showed a clear difference in protein expression patterns between *M. thermautotrophicus* delta H^T and *M. marburgensis* DX01 in response to different temperatures (Figure 4A). We noticed that the protein expression profiles of *M. thermautotrophicus* delta H^T were similar at the different culture temperatures. In contrast, the protein expression profiles of *M. marburgensis* DX01 at 70°C were observably different to the profiles at 50 and 60°C. This suggests that *M. marburgensis* DX01 may be more sensitive to temperature than *M. thermautotrophicus* delta H^T. It appears that more regulation of protein expression occurred under the different growth temperatures. Through N-terminal protein sequencing, we examined a protein that was induced at 70°C; this protein was the Methyl-coenzyme M reductase I, gamma subunit. Methyl-coenzyme M reductase (MCR), the key enzyme involved in methane formation, is a 300-kDa protein organized as a hexamer in an $\alpha_2\beta_2\gamma_2$ arrangement. This protein is thought to be required for all methanogenic pathways, and MCR expression can be seen to be highly increased at high temperature (Ermler et al., 1997; Rother et al., 2005). The amino acid sequence of MCR was highly conserved, with a 97.6% amino acid sequence identity between *M. thermautotrophicus* delta H^T and *M. marburgensis* DX01 (Figure 4C). Under extremely high temperatures, *M. marburgensis* DX01 may gain more energy through induction of MCR expression.

Therefore, the data presented here demonstrates that the genomes of the two *Methanothermobacter* strains

possess substantial genomic heterogeneity according to the PCR melting profile analysis. The new isolate of thermophilic methanogenic archaea, *M. marburgensis* DX01, possesses unique DNA fragments. To a certain extent, the results provided direct molecular evidence demonstrating why the morphology and protein fingerprint profiles between the two *Methanothermobacter* strains were different, although the two species exhibited extremely similar physiological characteristics. These studies may provide clues for better understanding the new isolate of thermophilic methanogenic archaea.

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