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Identification of the gene for disaggregatase from *Methanosarcina mazei*

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Summary The gene sequences encoding disaggregatase (Dag), the enzyme responsible for dispersion of cell aggregates of *Methanosarcina mazei* to single cells, were determined for three strains of *M. mazei* (S-6^T, LYC and TMA). The *dag* genes of the three strains were 3234 bp in length and had almost the same sequences with 97% amino acid sequence identities. Dag was predicted to comprise 1077 amino acid residues and to have a molecular mass of 120 kDa containing three repeats of the DNRLRE domain in the C terminus, which is specific to the genus *Methanosarcina* and may be responsible for structural organization and cell wall function. Recombinant Dag was overexpressed in *Escherichia coli* and preparations of the expressed protein exhibited enzymatic activity. The RT-PCR analysis showed that *dag* was transcribed to mRNA in *M. mazei* LYC and indicated that the gene was expressed in vivo. This is the first time the gene involved in the morphological change of *Methanosarcina* spp. from aggregate to single cells has been identified.

Keywords: methanochondroitin, morphological change.

Introduction

Methanosarcina spp. are the only members of methanogenic archaea that exhibit multiforms, namely aggregate (multicellular form) and single (unicellular form) cells (Zhilina 1976, Zhilina and Zavarzin 1979, Mah 1980, Robinson 1986, Mayerhofer et al. 1992). Aggregates are the forms that connect single cells with the heteropolysaccharidal extracellular matrix, the "methanochondroitin" (König 1988). Methanochondroitin is composed of galactosamine and glucuronic (or galacturonic) acid as well as minor amounts of glucose and a trace amount of mannose, which resembles eukaryotic chondroitin in chemical composition (Kreisl and Kandler 1986). The life cycle of *Methanosarcina mazei* was reported to switch between aggregated and single cells based on electron microscopic observation (Robinson 1986). Growth substrates (Boone and Mah 1987, Harris 1987), elevated concentrations of divalent cations (Boone and Mah 1987, Xun et al. 1988) and inoculum size (Xun et al. 1988) induce the spontaneous disaggregation of *M. mazei*.

Methanogenic archaea play an important role in the final decomposition process of organic matter and the production of a greenhouse gas, methane, in anoxic environments such as paddy fields. Because acetate is the precursor of about two-thirds of the methane produced on the earth (Boone 1991), acetoclastic methanogens (*Methanosarcina* and *Methanosaeta*) contribute significantly to the production of this greenhouse gas. *Methanosarcina* spp. are metabolically versatile, being able to use H_2/CO_2, acetate, methylamines and methanol as substrates for methanogenesis, and they have been isolated from various environments (Boone and Mah 2001). Therefore, it is important to understand the ecology and physiology of *Methanosarcina* spp. to better understand the mechanism of methanogenesis in the environment. The importance of the interconversion between cell aggregates and single cells is indicated in the ecophysiology of *Methanosarcina* spp. under various environmental conditions (e.g., Liu et al. 1985, Robinson 1986), but there are few studies on the mechanism underlying these morphological changes of *Methanosarcina* spp.

The transformation from cell aggregates to single cells depends on the enzyme disaggregatase (Dag) (Liu et al. 1985). The N-terminal amino acid sequence of Dag was determined by Xun et al. (1990), but the *dag* gene sequence has not yet

been reported. Determination of the gene sequence may give insights into the mechanism of interconversion of *Methanosarcina* aggregates to single cells. The aim of this study was to identify and characterize *dag*. We also conducted heterologous expression studies of Dag in *Escherichia coli* to investigate the enzymatic activity and facilitate transcription analysis of the *dag* gene.

Materials and methods

Archaeal strains

Methanosarcina mazei LYC (=DSM 4556) and S-6^T (=DSM 2053^T) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). *Methanosarcina mazei* TMA (=DSM 9195) was from our own collection (Asakawa et al. 1995).

Media and growth condition

The basal medium used for strain cultivation and maintenance of an aggregate form was a modification of low-phosphate basal medium (LPBM) (Asakawa et al. 1995). Medium preparation and cultivation techniques under anoxic conditions have been described previously (Asakawa et al. 1995). Single cells of *M. mazei* were grown in 40 ml of SC2 medium (Clarens et al. 1993) with a slight modification in a serum bottle (120 ml) or 500 ml of medium in a serum bottle (1200 ml) sealed with a butyl rubber stopper for 3 weeks at 37 °C without shaking. The SC2 medium contained (per liter of distilled water) 0.4 g KH_2PO_4; 1 g NH_4Cl; 0.4 g $CaCl_2{\cdot}2H_2O$; 10 g $MgCl_2{\cdot}6H_2O$; 23.4 g NaCl; 2 g Bacto yeast extract (Difco; Becton, Dickinson and Company); 2 g Polypepton (Nihon Pharmaceutical, Tokyo, Japan); 10 ml methanol; 9 ml trace mineral solution (Morii et al. 1983); 10 ml vitamin mixture solution (Zeikus 1977); 0.5 ml 0.2% resazurin solution; 0.5 g L-cysteine hydrochloride$\cdot H_2O$; 0.5 g $Na_2S{\cdot}9H_2O$; and 4.8 g $NaHCO_3$. The gas phase was H_2–CO_2 (4:1, 203 kPa), and the pH was 7.0. Aggregated cells of *M. mazei* were grown in 40 ml of MS medium (0.4 g KH_2PO_4; 1 g NH_4Cl; 0.4 g $CaCl_2{\cdot}2H_2O$; 1 g $MgCl_2{\cdot}6H_2O$; 2 g Bacto yeast extract (Difco); 2 g Polypepton (Nihon Pharmaceutical); 10 ml methanol; 9 ml trace mineral solution (Morii et al. 1983); 10 ml vitamin mixture solution (Zeikus 1977); 0.5 ml 0.2% resazurin solution; 0.5 g L-cysteine hydrochloride$\cdot H_2O$; 0.5 g $Na_2S{\cdot}9H_2O$; and 0.8 g $NaHCO_3$, per liter of distilled water, pH 6.8). The gas phase was N_2.

DNA and RNA extraction

Genomic DNA was isolated and purified from strains S-6^T and TMA as described previously (Asakawa et al. 1995). Genomic DNA was isolated and purified from strain LYC according to the method of Sowers (1995). The DNA and RNA for RT-PCR were extracted in duplicate as follows: 25 mg (wet mass) of pelleted *M. mazei* single cells was mixed with 150 µl of QRL1 solution (QIAGEN-tip 20, QIAGEN) and 0.56 g of glass beads with a diameter 0.1 mm, and the tube was shaken horizontally at 5000 rpm for 60 s with a Mini-Beadbeater (Biospec Product). The suspension was then placed on ice for 5 min and centrifuged at 12,000 *g* for 1 min at 4 °C. The DNA and RNA were isolated from the supernatant and purified using a QIAGEN-tip 20 (QIAGEN). Total RNA was incubated with 10 U of DNase I (RNase-free) (TaKaRa Bio) in the presence of 10 U of RNase inhibitor (Promega) for 1 h at 37 °C

Cloning and sequencing of dag

Standard DNA manipulations were performed according to Sambrook et al. (1989). The degenerate oligonucleotide probes, 5′-AARACNCCNACNGCNCCNAT-3′ (20mer) and 5′-ATHGTNTAYGTNGCNGGNGAYGG-3′ (23mer), were synthesized as a sense strand based on the known N-terminal amino acids sequence of Dag (KTPTAPIVYVAGDG) (Xun et al. 1990). The 3′ end of the probes was labeled with digoxigenin (DIG; Roche Diagnotics) using the DIG Oligonucleotide 3′ end Labeling Kit (Roche Diagnotics) according to the manufacturer's protocol and used for the genomic Southern hybridization. Genomic DNA from strain S-6^T was digested with *Eco*RI (TaKaRa Bio) and separated electrophoretically on a 0.7% agarose gel. After the digestion products were transferred to a nylon membrane (Hybond N+; GE Healthcare Bio-Science) using a VacuGene XL Vacuum Blotting System (GE healthcare Bio-Science), the DNA fragments on the membrane were hybridized with the DIG-labeled probes and luminescent detection was conducted with a DIG Labeling and Detection Kit (Roche Diagnostics) according to the kit protocol. Because the DIG-labeled probes hybridized fragments of about 3.2 kbp, these fragments were extracted, purified using a GENECLEAN II kit (BIO 101, Vista, USA), and ligated into the dephosphorylated *Eco*RI site of the vector pUC19 (TaKaRa Bio) with a DNA Ligation Kit Ver. 2 (TaKaRa Bio) to create a library. The competent *E. coli* XLI-Blue cells (Stratagene) were transformed with the ligation mixture by electroporation with a Gene Pluser (BioRad) and inoculated onto SOB agar plates (Sambrook et al. 1989) containing ampicillin (100 µg ml^{-1}) on which 5 µl of isopropyl β-D-thiogalactopyranoside (IPTG; 200 µg ml^{-1}) and 50 µl of 5-bromo-4-chloro-3-inodolyl β-D-galactopyranoside (X-gal; 20 µg ml^{-1}) were spread per plate. In total, 304 white colonies were picked and the plasmid DNAs were prepared by the alkaline lysis method and subjected to screening by dot-blot hybridization on Hybond N+ nylon membranes with the DIG-labeled oligonucleotide probes. The recombinant plasmid carrying two 3.2-kbp inserts was selected from five positive colonies and digested with several restriction endonucleases to locate *dag*. The *Bam*HI 2.7-kbp fragment of the insert was subcloned into the *Bam*HI and *Eco*RI site of pUC19 and the nucleotide sequence was determined for both strands by the primer walking method. The sequence corresponding to the N-terminal amino acids of Dag was found, but the complete gene was not contained in the fragment. Then, the *Eco*RI-*Bam*HI fragment containing the N-terminus of *dag* was labeled with DIG using a DIG Labeling and Detection Kit (Roche Diagnotics) and subjected to genomic Southern hybridization with the digestion products of both *Bam*HI and *Hin*dIII (TaKaRa Bio). The DIG-labeled DNA hybridized a

fragment of about 5.3 kbp, and a library was constructed with this fragment and pUC19 as described above. After 171 white colonies in total were screened by colony and Southern hybridizations with the DIG-labeled DNA, a recombinant plasmid carrying a fragment of about 5.3 kbp was selected. The nucleotide sequence of the 5.3-kbp fragment was determined for both strands by the primer walking method as mentioned above. The fragments of *dag* of strains LYC and TMA were screened and isolated from genomic DNAs from the respective strains by colony and Southern hybridizations with the DIG-labeled *Bam*HI and *Hin*dIII fragment containing *dag* from strain S-6^T and about 5.3-kbp fragments from *Bam*HI and *Hin*dIII digestion products of DNAs from both strains, and sequenced as described above.

Nucleotide sequences of the *dag* genes were determined with an ABI 373S automated sequencer (Applied Biosystems) with a PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS (Applied Biosystems) or Thermo Sequenase Dye Terminator Cycle Sequencing Kit (GE Healthcare Bio-Science).

Heterologous expression of Dag in Escherichia coli

The *dag* gene was amplified from genomic DNA of strain LYC with the primers f-*Bam*HI2 (5′-TACGCGGATCCGTatgCCTACTGCT-3′) and r-*Hin*dIII2 (5′-TACCCAAGCTTcaATT GATATACTCCAGC-3′) containing start and stop codons, where the underlined positions represent the restriction sites of *Bam*HI and *Hin*dIII, respectively, using *PfuTurbo* Hotstart DNA polymerase (Stratagene). The amplified fragments were cloned into the pCR 4Blunt-TOPO using the sequencing version of Zero Blunt TOPO PCR Cloning Kit (Invitrogen). The sequence of *dag* was confirmed for a plasmid carrying the correct insert as described above. The digested fragments of the plasmid by *Bam*HI and *Hin*dIII were then subcloned into the dephosphorylated sites of the pET-39b(+) expression vector (Merck KGaA) digested with the same restriction enzymes.

Dag was expressed from *E. coli* BL21(DE3) pLysS (Merck KGaA) containing the expression plasmid, pET-39b(+)-*dag*. The *E. coli* transformants were grown at 37 °C in Luria-Bertani (LB) medium supplemented with 30 μg ml^{-1} of kanamycin and 34 μg ml^{-1} of chloramphenicol. When the optical density (OD 660 nm) of the culture was greater than 0.4, 1 mM IPTG was added and the culture was further grown at 37 °C for 13.5 h. Cells were harvested by centrifugation at 10,000 *g* for 10 min at 4 °C. A small-scale analysis was conducted to examine the distribution of target protein in whole-cell extracts, medium, and soluble and insoluble fractions of cytoplasm according to the pET System Manual's protocol (Merck KGaA). Separation on an SDS-PAGE containing a 7.5% acrylamide separating gel was performed at 200 V per gel for 40 min, using a Mini-PROTEAN 3 cell (Bio-Rad). Commercially available kits (HMW-SDS Electrophoresis Calibration kit and LMW Electrophoresis Calibration kit, GE healthcare Bio-Science) of molecular mass standards were used. To reveal the protein patterns, the SDS-PAGE gels were stained with Fluka Coomassie brilliant blue R-250 (Sigma-Aldrich) at 0.5% (w/v) for 20 min.

Preparation of Dag from Escherichia coli for enzymatic assay

Cell pellets harvested from recombinant *E. coli* cultures were suspended in SC2 medium and sonicated 10 times for 5 s with a sonicator 5203 PZ1 (Ohtake Works, Tokyo, Japan) at 100 W. The suspension was centrifuged at 10,000 *g* for 10 min at 4 °C, and the supernatant was made anaerobic by bubbling with N_2 for 15 min and keeping at 4 °C overnight. This supernatant was used as the crude enzyme solution for the subsequent experiments.

Dag activity in the crude enzyme solution was assayed as described by Xun et al. (1990) with some modifications. Positive controls were the culture supernatants of *M. mazei* LYC (1st trial) and TMA (2nd trial) grown as single cells in SC2 medium and negative controls were the culture supernatants of strains LYC and TMA grown as aggregates in MS medium. The substrates for the assay were aggregated cells of strain S-6^T that were immersed in boiling water for 5 min to inactivate inherent Dag. The enzyme reaction mixture contained 0.5 ml of K-P buffer (1 M K_2HPO_4, 1 M KH_2PO_4, 0.02% sodium azide, 0.1% 2-mercaptoethanol, 0.0001% resazurin solution; pH 7.5), approximately 0.03 g, wet mass of substrate and 0.5 ml of crude enzyme solution. The enzyme reaction mixture was incubated for 2 h at 37 °C, and separation of aggregates into single cells was estimated from the turbidity (OD 660 nm) of the cell suspension.

Sequence analysis

Searches for homologs/orthologs of Dag (*M. mazei* LYC, S-6^T, and TMA) were performed with Protein-protein BLAST (blastp) and Position-specific iterated and pattern-hit initiated BLAST (PSI- and PHI-BLAST) search programs at NCBI (http://www.ncbi.nlm.nih.gov/), and the Pfam program at the Sanger Institute (http://www.sanger.ac.uk/Software/Pfam/). Proteins containing a disaggregatase related repeat (Disaggr_repeat; Pfam accession number PF06848) or disaggregatase related (Disaggr_assoc; Pfam accession number PF08480) domains, or both, were selected. The sequences were aligned and a phylogenetic tree was constructed with ClustalX and njplot programs, using the neighbor-joining method. Bootstrap analyses were performed with 1000 replications.

Reverse transcription PCR (RT-PCR)

Reverse transcription was carried out using ExScript RT reagent Kit (TaKaRa Bio). The RT reaction mixture (10 μl) contained *dag* 3′-end-specific primer (*dag*3214r, CAATTGATATA CTCCAGCTG, 2 pmol μl^{-1}), 2 μl of 5× ExScript Buffer (TaKaRa Bio), 0.5 μl of dNTP Mixture (10 mM each, TaKaRa Bio), 0.25 μl of ExScript RTase (200 U μl^{-1}, TaKaRa Bio), 0.25 μl of RNase Inhibitor (40 U μl^{-1}, TaKaRa Bio), and 1 μl of RNA extract. The RT reaction was performed at 42 °C for 20 min, followed by deactivation at 95 °C for 2 min.

The PCR mixture (50 μl) contained forward (*dag*18f, ATGCCTACTGCTTTGTGT) and reverse (*dag*3214r) primers (50 pmol μl^{-1} each), 2.5 U of *Ex-Taq* polymerase (TaKaRa

Bio), 5 µl of 10× *Ex-Taq* buffer (with 20 mM Mg^{2+}; TaKaRa Bio), 5 µl of dNTP mixture (2.5 mM each; TaKaRa Bio) and 1 µl of reverse transcription products. The PCR amplification was performed using a TaKaRa PCR Thermal Cycler (TaKaRa Bio). Amplification conditions were as follows: 95 °C for 2 min (initial denaturation), followed by 40 cycles of 95 °C for 0.5 min, 56 °C for 0.5 min and 72 °C for 3 min with a final extension at 72 °C for 10 min.

The PCR product was observed on agarose gel (0.7%) with 1× TAE buffer (40 mM Tris-HCl, 40 mM acetate, 1.0 mM EDTA) with ethidium bromide (0.5 µg ml^{-1}) under UV light.

Nucleotide sequence Accession numbers

The sequences of *dag* of the three *M. mazei* strains in this study are available from the DDBJ database. The Accession nos. are AB036793 (S-6^T), AB052161 (TMA) and AB052162 (LYC).

Results and discussion

Cloning of the putative dag, heterologous expression of Dag in Escherichia coli and its activity

The ORFs that we isolated from three strains (LYC, S-6^T and TMA) of *M. mazei* (putative *dag*) were 3234 bp and ran from an ATG codon to a TAA codon. The predicted mature proteins, based on the gene sequence data, were composed of 1077 amino acids, which included the identical sequence to the N-terminal sequence of Dag determined by Xun et al. (1990).

To confirm that the proteins encoded by the ORFs have disaggregating activity, the expression of protein was examined in the recombinant *E. coli*. The ORF of *M. mazei* LYC was placed under the control of the T7 promoter in the pET-39b(+) expression vector, and overexpressed in *E. coli* BL21 (DE3) pLysS. SDS-PAGE (Figure 1) demonstrated the selective expression of a protein with a molecular mass of about 120 kDa in the soluble fraction of cytoplasm extracted from recombinant *E.coli* cells, which was in good agreement with the deduced molecular mass of 120 kDa for the mature protein of *M. mazei* LYC.

Disaggregating activity was assayed in the crude enzyme solution of heterologously expressed protein using whole cells of *M. mazei* S-6^T as the substrate. No disaggregatase activity could be detected by the quantitative uronic acid assay described by Xun et al. (1990). Crude enzyme solution containing heterologously produced protein showed disaggregatase activity, although the increase in turbidity was only about twice that of the controls (Lanes 5–7 in Figure 2) because of the inherently high turbidity of the solutions extracted from *E. coli* cells. We concluded that the ORFs are *dag* that encodes Dag.

Xun et al. (1990) reported that purified Dag was insensitive to O_2 though a crude enzyme solution from the culture supernatant of *M. mazei* LYC lost activity on exposure to air. In our study, heterologous Dag was expressed and extracted under oxic conditions, and then reduced with Na_2S in an N_2 atmosphere overnight. These findings suggested that Dag exhibits

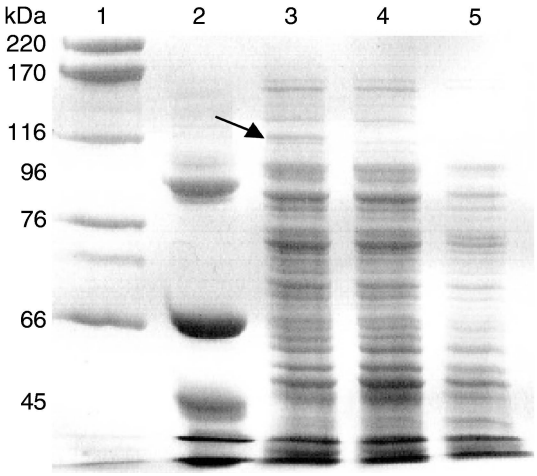

Figure 1. SDS-PAGE patterns of proteins in the soluble fractions extracted from recombinant cells of *Escherichia coli* BL21(DE3)pLysS. Arrow indicates heterologously expressed disaggregatase protein based on molecular mass. Lane 1, molecular mass marker (high molecular mass); Lane 2, molecular mass marker (low molecular mass); Lane 3, soluble fractions of induced cells containing the expression plasmid (pET-39b(+)-*dag*); Lane 4, soluble fractions of uninduced cells containing the expression plasmid; and Lane 5, soluble fractions of induced cells containing the expression vector (pET-39b(+)).

enzymatic activity only under anoxic conditions, but is tolerant to oxic conditions. Similar findings were reported for pseudomurein endoisopeptidase (Pei), which degrades cell walls of methanogenic archaeal members in Methanobac-

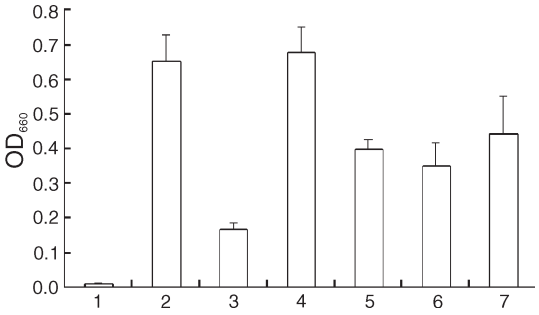

Figure 2. Disaggregating activity of soluble fractions extracted from recombinant *Escherichia coli* BL21(DE3)pLysS. The activity was determined with aggregated cells of strain S-6^T as substrate in K-P buffer by the turbidimetric assay as described in the Materials and methods. Blank, only substrate; 1, culture supernatants of *Methanosarcina mazei* grown as single cells with substrate (positive control); 2, culture supernatants of *M. mazei* grown as aggregates (negative control) with substrate; 3, soluble fractions of induced cells containing the expression plasmid (pET-39b(+)-*dag*) with substrate; 4, soluble fractions of uninduced cells containing the expression plasmid with substrate; 5, soluble fractions of induced cells containing the expression plasmid without substrate; and 6, soluble fractions of uninduced cells containing the expression plasmid without substrate. Values for the positive and negative controls are shown as averages with aggregated cells of *M. mazei* LYC (1st trial) and TMA (2nd trial). Bar indicates the range of the values obtained from two trials.

teriales and Methanopyrales, by Pfister et al. (1998). We used SC2 medium for preparation of the crude enzyme solution instead of the original K-P buffer (pH 7.5) (Xun et al. 1990) because Dag activity was recovered only for the preparation in SC2 medium. Some constituents in the SC2 medium may be essential for Dag activity, but the details remain to be elucidated.

Disaggregatase gene

The mature proteins of Dag of the three strains of *M. mazei* predicted from the *dag* sequence data showed almost the same sequence with 97% similarity to each other based on amino acid sequence. The molecular mass of Dag was 120,478 Da with a pI of 5.05, 120,699 Da with a pI of 5.08 and 120,541 Da with a pI of 5.04 for strains LYC, S-6^T and TMA, respectively. Xun et al. (1990) suggested that the Dag from strain LYC was a dimer of 77 to 80 kDa subunit, whereas Conway de Macario et al. (1993) reported that the molecular mass of Dag was 94 kDa as a monomer. The reason for the differences in the molecular masses of Dag between those studies and the present study is not clear.

Comparison of the nucleotide and amino acid sequences of *dag* and Dag with those in the GenBank database revealed no significant homology to other genes with known functions. The exceptions were several putative proteins of *M. mazei* Gö1 (Deppenmeier et al. 2002), *Methanosarcina acetivorans* C2A (Galagan et al. 2002) and *M. barkeri* fusaro (Maeder et al. 2006), whose complete genome sequences are available, with identities from 22 to 99%. Dag contained three tandem repeats of 200 amino acid residues in the C terminus. Adindla et al. (2004) compared the protein sequences of the ORFs of *M. acetivorans* C2A, *M. mazei* and *M. barkeri* with each other to systematically identify and analyze all sequence repeats in the cell surface proteins and found that one to three repeats are often found in Dag-like proteins and their relatives. They referred to the repeats as a DNRLRE domain based on the conserved sequence motif of the repeats and showed that the domain was characterized by consensus secondary structures and domain architectures (Adindla et al. 2004). The N-terminal half of Dag contained the conserved domain, "disaggr_assoc" (Pfam Accession no. PF08480), which seems to be the catalytic site. A schematic structure of Dag is shown in Figure 3a. The "disaggr_assoc" and DNRLRE ("disaggr_repeat" in the Pfam database with the Accession no. PF06848) families contain 32 and 19 proteins in *M. mazei, M. acetivorans* and *M. barkeri*, respectively, and details of the domains information are available in the Pfam database. Phylogenetic relationships among Dag of strains LYC, S-6^T and TMA together with all of the hypothetical proteins found in the genomes of *M. acetivorans* C2A, *M. mazei* Gö1 and *M. barkeri* fusaro which contain both of the "disaggr_assoc" and "disaggr_repeat" domains based on the Pfam database are shown in Figure 3b. Identity values ranged from 40 to 99% among the sequences. Dag of strains LYC, S-6^T and TMA and *M. mazei* Gö1 ORF number MM1144 (GenBank Accession no. NP_633168), which had 1095 amino acids and one

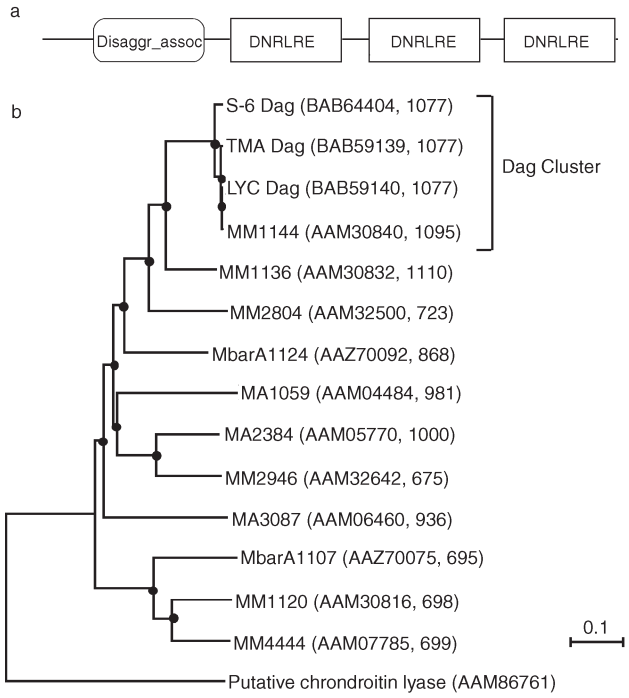

Figure 3. (a) Schematic structure of disaggregatase. Disaggr_assoc (200 residues) and DNRLRE (disaggr_repeat; 181 or 185 residues) domains are conserved motifs with Pfam accession nos.of PF08480 and PF06848, respectively. (b) Phylogenetic relationships between disaggregatase and the hypothetical proteins containing both of disaggr_assoc and disaggr_repeat domains found in genomes of *Methanosarcina mazei* Gö1, *M. acetivorans* C2A and *M. barkeri* fusaro based on amino acids sequences. Black circle at branch points indicates bootstrap value greater than 500 out of 1000 replicates. Chondroitin lyase (GenBank accession no. NP_670510) was used as an outgroup. The scale bar represents 0.1 substitutions per amino acid. Sequences are shown with their ORF numbers (MM, *M. mazei* Gö1 ORF; MA, *M. acetivorans* C2A ORF; MbarA, *M. barkeri* fusaro ORF). Protein ID and number of amino acid residues of protein are shown in parentheses.

"disaggr_assoc" and three "disaggr_repeat" domains, showed close relationships (97–99% identity) and formed a cluster. Cluster formation was also found in the phylogenetic trees constructed separately with regions of the domains "disaggr_assoc" (Supplementary Figure S1) and "disaggr_repeat" (Supplementary Figure S2). These findings indicate that MM1144 is a possible Dag of *M. mazei* Gö1 (alignment of amino acid sequences of the four proteins is shown in Supplementary Figure S3) and *dag* is conserved in *M. mazei* strains. The remaining ten proteins encoded by the ORFs found in genomes of *M. acetivorans* C2A, *M. mazei* Gö1 and *M. barkeri* fusaro contained a "disaggr_assoc" and at least one "disaggr_repeat" domain and showed sequence identities with 42 to 77% to Dag and possible Dag (MM1144) proteins. Those putative proteins may be homologs or orthologs of Dag in *Methanosarcina* spp. Further investigation is needed to elucidate the functions of these proteins.

Adindla et al. (2004) reported that the DNRLRE domain is specific to the genus *Methanosarcina* and indicated that it may

be responsible for structural organization and cell wall function. Pseudomurein endoisopeptidase (Pei) hydrolyzes pseudomurein, a cell wall constituent of methanogenic archaeal members in Methanobacteriales and Methanopyrales, and is another cell wall degrading enzyme in methanogenic archaea. Pseudomurein cell wall binding domains were recently identified and characterized in Pei proteins and three repeats of the domains were found in the N-terminus of the PeiW and PeiP (Steenbakkers et al. 2006). Further investigation is needed to elucidate the functions of the DNRLRE ("disaggr_repeat") and "disaggr_assoc" domains in relation to interconversion by Dag.

Transcription analysis of dag in M. mazei LYC

Total RNA was extracted from *M. mazei* LYC culture grown as single cells, the supernatant of which exhibited disaggregating activity (data not shown). The RT-PCR was strictly dependent on the absence of DNA because no *dag* PCR signal was observed when the reverse transcriptase was absent (Lane N in Figure 4). The 3.2-kbp RT-PCR product estimated from a DNA molecular mass standard was detected in a 0.7% agarose gel by electrophoresis (Figure 4), indicating that the size of the transcripts was same as that of *dag*. This finding confirmed that *dag* was monocistronically transcribed in *M. mazei* LYC.

In conclusion, the gene responsible for disaggregation of *M. mazei* was identified for the first time. The relationships among expression of *dag*, morphology and methane emission remain to be elucidated. Our study provides further understanding of the interconversion between *Methanosarcina* spp. at the molecular and genetic levels, and also of the ecophysiology of *Methanosarcina* spp.

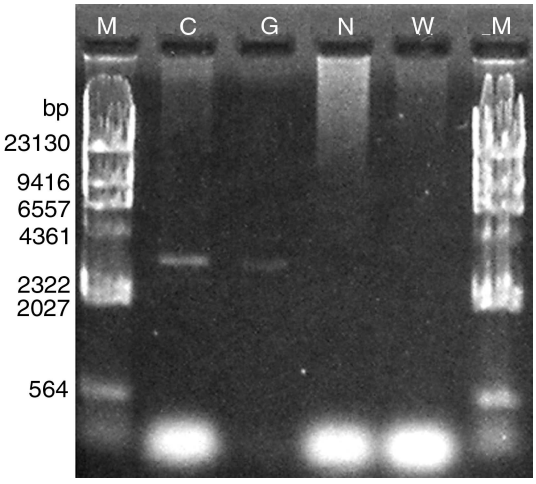

Figure 4. Agarose gel electrophoresis of RT-PCR products from mRNA of *Methanosarcina mazei* LYC with the *dag* gene specific primer set. C, PCR products from cDNA after RT reaction with mRNA extracted from single cells of *M. mazei* LYC; G, PCR products from genomic DNA of *M. mazei* LYC; N, PCR products without RT reaction from DNA-free RNA solution extracted from single cells of *M. mazei* LYC (negative control); W, PCR products from MilliQ-Water; and M, molecular mass standard (λ*Hin*dIII).

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Supplementary material

Figure S1. Phylogenetic relationships among motifs of disaggr_assoc domain found in *Methanosarcina mazei*, *M. acetivorans* and *M. barkeri* based on amino acids sequences. Available at:

http://archaea.ws/archive/data/volume2/2-185/2-185.FigureS1.pdf

Figure S2. Phylogenetic relationships among motifs of disaggr_repeat domain found in *Methanosarcina mazei*, *M. acetivorans* and *M. barkeri* based on amino acids sequences. Available at:

http://archaea.ws/archive/data/volume2/2-185/2-185.FigureS2.pdf

Figure S3. Alignment of protein sequences of disaggregatase from *Methanosarcina mazei* LYC, S-6^T and TMA and ORF MM1144 (possible disaggregatase) of *M. mazei* Gö1. Available at:

http://archaea.ws/archive/data/volume2/2-185/2-185.FigureS3.pdf