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Pseudomurein endoisopeptidases PeiW and PeiP, two moderately related members of a novel family of proteases produced in *Methanothermobacter* strains

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Abstract

Sequence comparison of pseudomurein endoisopeptidases PeiW encoded by the defective prophage ΨM100 of *Methanothermobacter wolfeii*, and PeiP encoded by phage ΨM2 of *Methanothermobacter marburgensis*, revealed that the two enzymes share only limited similarity. Their amino acid sequences comprise an N-terminal domain characterized by the presence of direct repeats and a C-terminal domain with a catalytic triad C-H-D as in thiol proteases and animal transglutaminases. Both PeiW and PeiP catalyze the in vitro lysis of *M. marburgensis* cells under reducing conditions and exhibit characteristics of metal-activated peptidases. Optimal temperature and pH were determined to be 63°C and 6.4 for His-tagged PeiP and 71°C and 6.4 for His-tagged PeiW, respectively. Database search results suggest that PeiW and PeiP are the first two experimentally identified members of a novel family of proteases in a superfamily of archaeal, bacterial, and eukaryotic protein homologs of animal transglutaminases.

Keywords: Archaeon; *Methanothermobacter*; Enzyme characterization; Pseudomurein endoisopeptidase; Metal-activated protease

1. Introduction

Besides proteasomes [1], several non-methanogenic Archaea are also known to produce extracellular proteases belonging to different classes, including (i) serine proteases produced by a number of halobacteria [2] and *Sulfolobus solfataricus* [3]; (ii) subtilisin-like proteases such as the one bound to the surface layer of *Staphylothermus marinus* [4]; (iii) thiol proteases, e.g. from *Pyrococcus kodakaraensis* KOD1 [5]; (iv) a maltose-regulated prolyl oligopeptidase from *Pyrococcus furiosus* [6]; (v) metalloproteases such as *S. solfataricus* zinc carboxypeptidase [7] and *P. furiosus* cobalt-activated carboxypeptidase [8]. In addition, a number of yet unclassified archaeal proteases were also reported, such as *Thermus aquaticus* caldolysin [9].

Not much is known about proteases in methanogens. Two lytic enzymes degrading the pseudomurein cell wall sacculi found in members of the order Methanobacteriales have been described in *Methanothermobacter wolfeii* (formerly *Methanobacterium wolfei*) [10,11] and in phage ΨM2 of *Methanothermobacter marburgensis* (formerly *Methanobacterium thermoautotrophicum* Marburg) [11,12]. The former is responsible for autolysis of *M. wolfeii* under hydrogen limitation [13] and was recently shown to be encoded in the genome of defective prophage ΨM100 [14]. Both enzymes cleave isopeptide bonds formed between the ε-amino group of an L-lysine residue and the α-carboxyl group of an L-alanine residue in the oligopeptides linking sugar chains of pseudomurein [10]. Because of their unique substrate specificity, pseudomurein endoisopeptidases are likely to belong to a new family of proteases. In the present work, we report the sequence comparison, overexpression, and characterization of the purified enzymes.

2. Materials and methods

2.1. Sequence analysis methods

Computer analysis of sequence data was carried out with the GCG software package. Searches for homologs

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of the pseudomurein endoisopeptidases PeiW and PeiP were performed with BLAST at the NCBI [15].

2.2. Cloning, expression and purification of enzymes

The cloned *peiP* gene [12] was recloned into the expression vector pET28a and the resultant construct was designated pME2508. The gene of the autolytic enzyme PeiW was amplified by PCR using the primers PeiWFor (5′-AGGTGATCATATGGAAGTGG-3′, *Nde*I site underlined) and PeiWRev (5′-AACAACTCGAGCATGTCTC-3′, *Xho*I site underlined) as a 0.9-kb *Nde*I–*Xho*I fragment from the *M. wolfeii* genomic DNA. It was then cloned into pUC19 digested with *Nde*I and *Sal*I to give plasmid pME2557. After verifying the sequence, the *peiW* gene was recloned as a 0.9-kb *Nde*I–*Hin*dIII fragment into the expression vector pET28a, resulting in plasmid pME2558.

For expression of the pseudomurein endoisopeptidase PeiW and PeiP, *Escherichia coli* BL21(DE3) cells harboring the respective expression construct alone or together with the helper plasmid pME2502 [16] were grown in LB medium in the presence of the corresponding antibiotic(s) at 30°C to an OD_{600} of 0.5, and induced by addition of IPTG to a final concentration of 25 μM and further incubated for another 8 h. The cultures were harvested by centrifugation at $5000 \times g$ and 4°C for 10 min, suspended in a suitable volume of 20 mM potassium phosphate buffer (pH 7.0). The samples were then disrupted by three passages through a French press cell at ca. 90 MPa. Clarified crude extracts were obtained after 10 min centrifugation at $16\,000 \times g$ and 4°C. Without prior reactivation in a reduced buffer, they were directly used for enzymatic assays to monitor the cell wall-degrading activity as described [12].

Purification of native PeiW from autolysates of *M. wolfeii* was performed according to the method described previously [10]. After overexpression as N-terminally His-tagged derivatives in *E. coli*, both PeiW and PeiP were purified to homogeneity by Ni-NTA affinity chromatography under native conditions. They were further filtered with Ultrafree-4 Centrifugal Filter Units Biomax-50 with a nominal molecular mass limit of 10 kDa (Millipore) and the buffer was eventually replaced by 20 mM potassium phosphate buffer (pH 7.0).

2.3. Enzyme characterization, inhibition and reactivation studies

To determine their optimal pH and temperature, the purified enzymes were preincubated overnight at 4°C under 2 bar H_2/CO_2 (80:20, v/v) in the presence of 30 mM dithiothreitol (DTT) and 2 mM $MgCl_2$. All cell suspensions of *M. marburgensis* used were also preincubated as previously described [12]. The optimal temperatures were measured using 10^8 cells of *M. marburgensis* suspended in 1 ml 50 mM potassium phosphate buffer (pH 7.0). Optimal pH values were determined at the optimal temperature for all enzymes with *M. marburgensis* cells suspended in various buffer solutions including 50 mM KH-phthalate, 50 mM potassium phosphate, or 50 mM Tris–HCl. All buffer solutions were prepared according to Dawson et al. [17] and pH values ranging from 5 to 8.5 at the respective optimal temperatures were set with a pH meter. One enzyme unit (U) is defined as the activity of an amount of protein that leads to a 50% decrease in optical density at 546 nm in 1 h.

The effects of ethylenediaminetetraacetic acid (EDTA) and of divalent cation(s) on enzyme activity were determined under conditions of both optimal pH and temperature. EDTA pretreatment was performed by incubating the enzymes with EDTA (final concentration 100 mM) for 2 h in an anaerobic chamber. EDTA was then removed by using Ultrafree-4 Centrifugal Filter Units Biomax-50 with a nominal molecular mass limit of 10 kDa (Millipore) and the buffer was replaced by 20 mM potassium phosphate buffer (pH 7.0). The enzyme preparations were then prereduced overnight at 4°C under 2 bar H_2/CO_2 (80:20, v/v) in the presence of 30 mM DTT but no $MgCl_2$. The remaining enzyme activity was determined with prereduced 10^8 cells of *M. marburgensis* suspended in 50 mM potassium phosphate buffer at the respective optimal pH. Reactivation of EDTA-pretreated enzymes by divalent cations was performed in unbuffered solutions of $CaCl_2$ or $MgCl_2$ to a final concentration of 2 mM. Cell suspensions of *M. marburgensis* in 50 mM potassium phosphate buffer (for $MgCl_2$) or 50 mM Tris–HCl buffer (for $CaCl_2$) reduced by 30 mM DTT were used.

3. Results and discussion

3.1. Comparison of pseudomurein endoisopeptidases PeiP (M. marburgensis) and PeiW (M. wolfeii)

BLAST searches [15] suggested that the prophage ΨM100-encoded PeiW is most similar to PeiP of phage ΨM2 (probability with BLAST: 4×10^{-83}). Sequence comparison of *peiW* and *peiP* revealed that the two enzymes are only moderately related and share only 53.4% identity at the amino acid sequence level. Two distinct domains could be identified in their sequence (Fig. 1). The N-terminal domain is characterized by the presence of direct repeats. In PeiP, two copies of a nearly perfect direct repeat of 69 amino acids were present, while in PeiW, there are two complete copies of a perfect direct repeat of 28 amino acids and a partial copy of 20 amino acids between the two perfect repeats. The repeats can also be found in the nucleotide sequence of gene *peiW,* but not in that of gene *peiP*. Repeats are consistent with the polymer nature of the substrate, pseudomurein, and may serve as a determinant of substrate specificity. The C-terminal domain of both isopeptidases possesses a catalytic triad of three ami-

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                .         .         .         .         .
PeiP     MRSNSVNIETFKDMLKRYEDFKMKNKREPRVIFIRSGGGESIPLETFRDM 1-50
             .|.::.| || |||||||||||||||  :: . .| .: |..|:||
PeiW     ...MEVGLNEFLDMKKRYEDFKMKNKREPR..YVTTKNGYKVMLPVFKDM 1-45
                .         .         .         .         .
PeiP     VRRYNNFKDRYGREPRIVYVTPPEPPVPEVNENTPEYVSITQFKDMLSRY 51-100
         :|||::|    ||||..: : |          .. : :.|..|:|||.||
PeiW     LRRYEDFVRINGREPNYISIQP.........QPNGKIEIKKFRDMLRRY 46-85
                .         .         .         .         .
PeiP     NRFKEVNGREPRVVFIYSGGGPSVSLETFKDMCKRYNQFLEENRREPRIV 101-150
         : |  :|||||.::::  | :. |||:|||| :||.:|:  | |||..:
PeiW     EDFVRINGREPNIIYLEQGKSDHVSLGTFKDMLRRYKDFVRINGREPNYI 86-135
                .         .         .         .         .
PeiP     YVTPPEPPVPEEVREMRRVLGEFKTATQLYTLVSRRCKYKFYYNDQTPNR 151-200
          : |...  .. . .:   :|.|..|| ||. |.: ||||:|||||.||:
PeiW     SIQPQPSLKGHWTTKVIEKIGTFHDATSLYERVKKTCKYKYYYNDQVPNH 136-185
                .         .         .         .         .
PeiP     EALKKMVTDGINCTDACQLFKPVIEGLGYSVRIEHVKVRCNDNKWYGHYF 201-250
          |:.:|.|.|||||||||||..|:|::||.|:||||:|:|||.||||||:
PeiW     VAVMRMTTSGINCTDACQLFSKVLEEMGYEVKIEHVRVKCNDGKWYGHYL 186-235
                .         .         .         .         .
PeiP     LRVAGKELASVSLPSERWTVWDYVSATKTGRPLGAPCCSRGIQHLGWGIV 251-300
         |||:| || ..       |:||||||||||||.|||. |:||||||||
PeiW     LRVGGFELKDG.......TIWDYVSATKTGRPLGVPCCTAGFQHLGWGIV 236-278

PeiP     SPKHD  301-305
         :| .|
PeiW     GPVYDK 279-284
```

Fig. 1. Protein sequence alignment of the pseudomurein endoisopeptidases PeiP and PeiW generated with the GAP program of GCG. The direct repeats in their sequences and the catalytic triad C-H-D are highlighted.

no acids, namely a cysteine, a histidine and an aspartate. The same catalytic triad is also present in animal transglutaminases like human blood clotting factor XIII and thiol proteases such as papain [18].

With a BLAST probability of 5×10^{-4}, PeiW is also homologous to MTH412 of *M. thermoautotrophicus* ΔH [19], and, at lower similarities, to some other members of family 6 in a superfamily of archaeal, bacterial, and eukaryotic protein homologs of animal transglutaminases, especially in the C-terminal catalytic region [18]. The pseudomurein in the cell wall of *Methanothermobacter* species is expected to be selectively degraded by yet uncharacterized enzyme system(s) in order to permit cell division. In the chromosome of *M. thermoautotrophicus* ΔH there are several open reading frames encoding proteins (e.g. MTH 412) that show similarities to PeiW and PeiP and may possess a pseudomurein lytic activity. This raises the possibility that phage ΨM2 once acquired the isopeptidase function from its sole known host, *M. marburgensis* [11].

3.2. Purification of native PeiW, overexpression and purification of PeiW and PeiP as N-terminal His-tagged derivatives

Native PeiW was purified from autolysates of *M. wolfeii* (data not shown) and both PeiW and PeiP with an N-terminal His tag were overexpressed in *E. coli* at significant levels and subsequently purified to homogeneity (Fig. 2). The *peiW* gene (encoding a polypeptide of 284 amino acids) contains 12 rare arginine codons (AGG/AGA) and six rare isoleucine codons (AUA), including one tandem repeat. The *peiP* gene (coding potential of 305 residues) contains 21 rare arginine codons (AGG/AGA), including one tandem repeat, and six rare isoleucine codons (AUA). In spite of the high percentage of those two rare codons, the presence of the helper plasmid pME2502 designed to compensate for codon usage bias in Methanobacteriales [16] had no apparent influence on the level of heterologous expression in *E. coli* (data not shown).

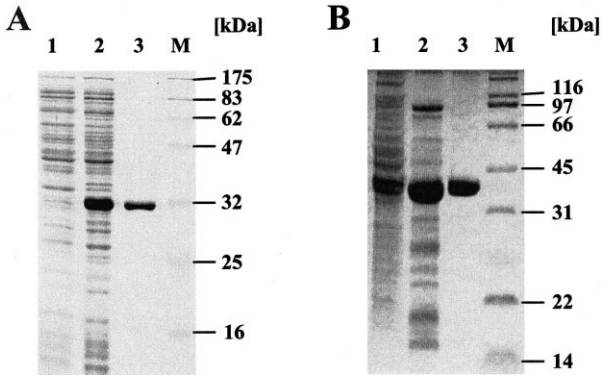

Fig. 2. Overexpression and purification of PeiW (A) and PeiP (B). A: Protein samples were loaded onto a 12% SDS–PAGE gel. Lane 1, clarified crude extract from induced *E. coli* BL21(DE3)(pET28a); lane 2, clarified crude extract from induced *E. coli* BL21(DE3)(pME2558); lane 3, the purified His-tagged PeiW. B: Protein samples were loaded onto a 10% SDS–PAGE gel. Lane 1, clarified crude extract from induced *E. coli* BL21(DE3)(pME2508); lane 2, partially purified His-tagged PeiP by SP Sepharose chromatography; lane 3, the purified His-tagged PeiP. M, molecular mass standards.

3.3. Properties of the enzymes, inhibition and reactivation by divalent metal ions

By using purified enzymes, some properties of the pseudomurein endoisopeptidases were determined semi-quantitatively using whole cells of *M. marburgensis* as a substrate. The optimal temperature for His-tagged PeiW was determined to be 71°C (Fig. 3A), which is higher than the optimal growth temperature (ca. 60°C) of *M. wolfeii*. It is also slightly higher than that of native PeiW (67°C, data not shown). The difference may be due to the presence of the N-terminal His tag. His-tagged PeiP exhibited maximal activity at a lower temperature (63°C, Fig. 3A). All enzymes assayed, i.e. native PeiW and the His-tagged PeiW and PeiP, had an optimal pH of 6.4 in several different buffer systems (Fig. 3B and data not shown).

As shown in Fig. 4A, EDTA inhibited the cell wall-degrading activity of PeiP and PeiW, and the activity of EDTA-pretreated enzymes could be largely recovered by addition of divalent cations like Ca^{2+} or Mg^{2+} (Fig. 4B). These data suggest that both PeiP and PeiW are metal-activated peptidases.

Pseudomurein endoisopeptidases PeiP and PeiW provide powerful tools for the study of pseudomurein-containing methanogens. For example, the partially purified native PeiW proved useful for the high-yield isolation of undegraded chromosomal and plasmid DNA from *M. marburgensis* and for the preparation of *Methanothermobacter* protoplasts [10]. In this respect, active recombinant pseudomurein endoisopeptidases offer significant advantages. However, currently available assays for the enzymes utilizing either whole cells [12] or purified cell walls [20] do have some limitations for quantitative measurements. An improved assay using synthetic peptides as substrates would significantly help to measure kinetic enzyme parameters and also allow the determination of minimal substrate requirements and substrate specificity.

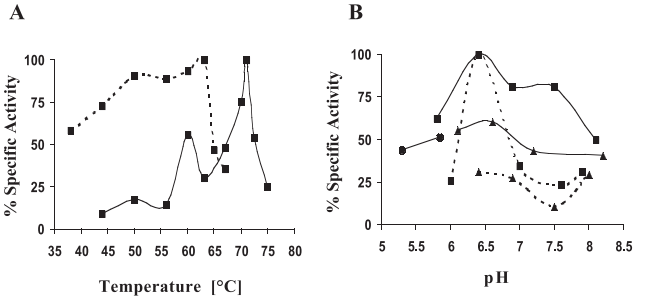

Fig. 3. The optimal temperature (A) and pH (B) of the N-terminally His-tagged pseudomurein endoisopeptidases PeiW (solid lines) and PeiP (dotted lines). For each assay, ca. 1.5 μg purified His-tagged PeiW or ca. 15 μg purified His-tagged PeiP was used. A: The measurement of activity under different temperatures was done with cell suspensions of *M. marburgensis* Marburg in 50 mM potassium phosphate (pH 7.0). 100% specific activity refers to 1800 U mg^{-1} for PeiW and 279 U mg^{-1} for PeiP. B: The activity was measured in 50 mM KH-phthalate buffer (dots), 50 mM potassium phosphate buffer (rectangles) and 50 mM Tris buffer (triangles). 100% specific activity refers to 960 U mg^{-1} for PeiW and 566 U mg^{-1} for PeiP. The data shown are representative of two or three independent experiments.

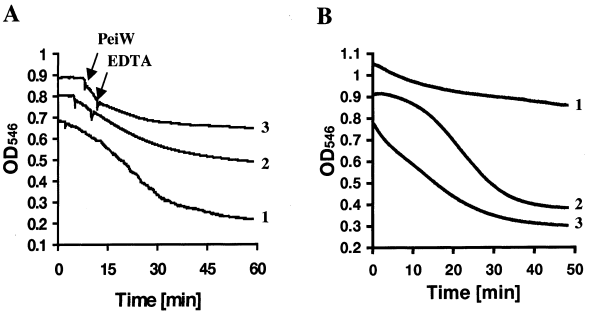

Fig. 4. The effect of EDTA and divalent cation(s) on the activity of His-tagged PeiW. A: Inhibition of EDTA of the cell wall-degrading activity of the N-terminally His-tagged PeiW. The arrows indicate the addition of PeiW and EDTA, respectively. 1, control; 2 and 3, EDTA added at a final concentration of 50 mM or 100 mM, respectively. B: Reactivation of EDTA-pretreated His-tagged PeiW. 1, control; 2 and 3, Ca^{2+} or Mg^{2+} added at time 0 at a final concentration of 2 mM, respectively. The results are representative of three independent experiments.

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References

[1] Dahlmann, B., Kopp, F., Kuehn, L., Niedel, B., Pfeifer, G., Hegerl, R. and Baumeister, W. (1989) The multicatalytic proteinase (prosome) is ubiquitous from eukaryotes to archaebacteria. FEBS Lett. 251, 125–131.

[2] Kamekura, M., Seno, Y. and Dyall-Smith, M. (1996) Halolysin R4, a serine proteinase from the halophilic archaeon *Haloferax mediterranei*: gene cloning, expression and structural studies. Biochim. Biophys. Acta 1294, 159–167.

[3] Burlini, N., Magnani, P., Villa, A., Macchi, F., Tortora, P. and Guerritore, A. (1992) A heat-stable serine proteinase from the extreme thermophilic archaebacterium *Sulfolobus solfataricus*. Biochim. Biophys. Acta 1122, 283–292.

[4] Mayr, J., Lupas, A., Kellermann, J., Eckerskorn, C., Baumeister, W. and Peters, J. (1996) A hyperthermostable protease of the subtilisin family bound to the surface layer of the archaeon *Staphylothermus marinus*. Curr. Biol. 6, 739–749.

[5] Morikawa, M., Izawa, Y., Rashid, N., Hoaki, T. and Imanaka, T. (1994) Purification and characterization of a thermostable thiol protease from a newly isolated hyperthermophilic *Pyrococcus* sp. Appl. Environ. Microbiol. 60, 4559–4566.

[6] Harwood, V.J., Denson, J.D., Robinson-Bidle, K.A. and Schreier, H.J. (1997) Overexpression and characterization of a prolyl endopeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Bacteriol. 179, 3613–3618.

[7] Colombo, S., D'Auria, S., Fusi, P., Zecca, L., Raia, C.A. and Tortora, P. (1992) Purification and characterization of a thermostable carboxypeptidase from the extreme thermophilic archaebacterium *Sulfolobus solfataricus*. Eur. J. Biochem. 206, 349–357.

[8] Cheng, T.C., Ramakrishnan, V. and Chan, S.I. (1999) Purification and characterization of a cobalt-activated carboxypeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. Protein Sci. 8, 2474–2486.

[9] Khoo, T.C., Cowan, D.A., Daniel, R.M. and Morgan, H.W. (1984) Interactions of calcium and other metal ions with caldolysin, the thermostable proteinase from *Thermus aquaticus* strain T351. Biochem. J. 221, 407–413.

[10] Kiener, A., König, H., Winter, J. and Leisinger, T. (1987) Purification and use of *Methanobacterium wolfei* pseudomurein endopeptidase for lysis of *Methanobacterium thermoautotrophicum*. J. Bacteriol. 169, 1010–1016.

[11] Wasserfallen, A., Nölling, J., Pfister, P., Reeve, J. and Conway de Macario, E. (2000) Phylogenetic analysis of 18 thermophilic *Methanobacterium* isolates supports the proposals to create a new genus, *Methanothermobacter* gen. nov., and to reclassify several isolates in three species, *Methanothermobacter thermautotrophicus* comb. nov., *Methanothermobacter wolfeii* comb. nov., and *Methanothermobacter marburgensis* sp. nov. Int. J. Syst. Evol. Microbiol. 50, 43–53.

[12] Pfister, P., Wasserfallen, A., Stettler, R. and Leisinger, T. (1998) Molecular analysis of *Methanobacterium* phage ΨM2. Mol. Microbiol. 30, 233–244.

[13] König, H., Semmler, R., Lerch, C. and Winter, J. (1985) Evidence for the occurrence of autolytic enzymes in *Methanobacterium wolfei*. Arch. Microbiol. 141, 177–180.

[14] Luo, Y., Pfister, P., Leisinger, T. and Wasserfallen, A. (2001) The genome of archaeal prophage ΨM100 encodes the lytic enzyme responsible for autolysis of *Methanothermobacter wolfeii*. J. Bacteriol. 183, 5788–5792.

[15] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

[16] Luo, Y., Leisinger, T. and Wasserfallen, A. (2001) Comparative sequence analysis of plasmids pME2001 and pME2200 of *Methanothermobacter marburgensis* strains Marburg and ZH3. Plasmid 45, 18–30.

[17] Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1969) Data for Biochemical Research, 2nd edn. Oxford University Press, New York.

[18] Makarova, K.S., Aravind, L. and Koonin, E.V. (1999) A superfamily of archaeal, bacterial, and eukaryotic proteins homologous to animal transglutaminases. Protein Sci. 8, 1714–1719.

[19] Smith, D.R., Doucette-Stamm, L.A., Deloughery, C., Lee, H., Dubois, J., Aldredge, T., Bashirzadeh, R., Blakely, D., Cook, R., Gilbert, K., Harrison, D., Hoang, L., Keagle, P., Lumm, W., Pothier, B., Qiu, D., Spadafora, R., Vicaire, R., Wang, Y., Wierzbowski, J., Gibson, R., Jiwani, N., Caruso, A., Bush, D., Safer, H., Patwell, D., Prabhakar, S., McDougall, S., Shimer, G., Goyal, A., Pietrokovski, S., Church, G.M., Daniels, C.J., Mao, J.-I., Rice, P., Nölling, J. and Reeve, J.N. (1997) Complete genome sequence of *Methanobacterium thermoautotrophicum* ΔH: functional analysis and comparative genomics. J. Bacteriol. 179, 7135–7155.

[20] Morii, H. and Koga, Y. (1992) An improved assay method for a pseudomurein-degrading enzyme of *Methanobacterium wolfei* and the protoplast formation of *Methanobacterium thermoautotrophicum* by the enzyme. J. Ferment. Bioeng. 73, 6–10.