Bacillus stearothermophilus Cell Shape Determinant Gene, mreC and mreD, and Their Stimulation of Protease Production in Bacillus subtilis

Motoki KUBO,† Dennis J. PIERRO, Yoshino MOCHIZUKI, Tatsuya KOJIMA, Takenori YAMAZAKI,‡ Sachiko SATOH,§ Noboru TAKIZAWA, and Hohzoh KIYOHARA

Department of Chemistry and Biochemistry, Numazu College of Technology, 3600 Ooka Numazu-shi, Shizuoka 410, Japan
†Department of Applied Chemistry, Faculty of Engineering, Okayama University of Science, 1–1 Ridai-cho, Okayama 700, Japan
‡Department of Applied Chemistry, Faculty of Engineering, Research Institute of Technology, Okayama University of Science, 1–1 Ridai-cho, Okayama 700, Japan

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Protease production stimulating genes were isolated from a soybean protein degrading bacterium, Bacillus stearothermophilus HA19. The cloned fragment stimulated production of a 37-kDa protease in B. subtilis. The nucleotide sequence of the genes and their flanking regions were identical to the B. subtilis cell shape determinant genes mreC and mreD [J. Bacteriol., 176, 6729–6742 (1992); J. Bacteriol., 176, 6717–6728 (1992)]. The mreC and mreD genes in B. subtilis stimulate secretion of a neutral protease (37-kDa), and the protease activity in the culture medium reached 2500 U per ml (approximately 10 times higher than the host strain) after 24 h of cultivation in L broth, suggesting the mreCD genes regulate protease expression and the protease is related to the cell shape determination in Bacilli. The protease productions in B. subtilis carrying mreC or mreD deletion plasmids were not elevated, so the 37-kDa protease stimulation requires both mreC and mreD genes. The extracellular protease was purified, and the molecular mass of the enzyme was 37,000 Da by SDS–polyacrylamide gel electrophoresis and gel filtration. The optimum pH and temperature for the enzyme activity were 7.0 and 50°C, respectively, and the enzyme was stable at pH 7–10. The enzyme was inactivated by EDTA, but not by phenylmethylsulfonyl fluoride and disopropyl fluorophosphate.

Key words: Bacillus stearothermophilus; mreC, D genes; cell shape determinant genes; protease production

The spore-forming bacterium Bacillus subtilis is an attractive organism for studying the problem of cell division because it has two alternative pathways of septum formation. During vegetative growth, the bacterium divides medially, a process that strongly resembles binary fission in Escherichia coli and many other bacteria. The mreBCD genes have been cloned from the Gram-positive rod-shaped bacterium Bacillus subtilis.4,5 The MreB protein shows significant amino acid sequence identity to its E. coli counterpart. The rodB1 mutation of B. subtilis is a temperature-sensitive mutation in mreD that results in the cells becoming spherical at nonpermissive temperatures.3 The cell shape determination by mre genes in B. subtilis, however, has not been analyzed completely.

On the other hand, the cell shape determination mre genes of Escherichia coli were first identified by deletion mutations that rendered the cells spherical and altered their sensitivities to the amidino penicillin mecillinam. Five genes of the mre operon have been sequenced completely (mreBCD-orfE-orfF).1–3 The mreB gene product has been postulated to be a negative regulator of ftsI gene expression.3 However, the functions of MreC, MreD, OrfE, and OrfF are unknown.

We have isolated soybean-waste degrading and protease producing bacteria, B. stearothermophilus HA19 and B. circulans HA12, and the soybean waste degradation products (DSP) with the protease producing microorganisms, B. stearothermophilus HA19 and B. circulans HA12, showed stimulatory effects against plants.6 The DSP contained abundant nitrogen compounds such as amino acids, peptides, and proteins. To analyze the protease specificity concerning degradation of proteins from plants, we tried to isolate genes participating in the protease production gene from B. stearothermophilus HA19. We report here the cloning and sequencing of the protease secretion stimulating genes from B. stearothermophilus HA19, and that the genes are 100% identical to the cell shape determinants gene, mreCD, from B. subtilis.4,5

Materials and Methods

Bacterial strains, plasmids and media. The Bacillus strains used were B. stearothermophilus,6 B. subtilis MT-2 (tpcC2, leucC7, hisD8, hisD8, Npr+),† B. subtilis M1113 (argC15, trpC2, hisR, hisM),7 and B. subtilis DB104 (his, nprC2, napC1, dnapA3).8 Escherichia coli K12 JM109 (rec A1, Muv-proL, endA1, gyrA96, thi-1, hsdR17, relA1, supE44, F' traD36, proAB+).9 The host strain for pUC19 was pBR322 (KmR),10 and pUC19 (Ap'). Bacteria were grown in L broth, on L agar, or on LC agar (L agar plus 1% casein).7 The antibiotics used were kanamycin (5 μg/ml) for B. subtilis and ampicillin (50 μg/ml) for E. coli.

Isolation of plasmid and chromosomal DNA, restriction enzyme treatment,igration of DNA and transformation. Plasmid DNA was prepared by the rapid alkaline extraction method or CsCl/ethidium bromide equilibrium

† To whom correspondence should be addressed.
density gradient centrifugation. Chromosomal DNA was prepared as described by Imanaka et al. Treatment of DNA with restriction enzymes and ligation of DNA with T4 DNA ligase was done as recommended by the manufacturer. For transformation of B. subtilis and E. coli with plasmid DNA, competent cells were prepared as described by Imanaka et al.

**Gel electrophoresis for DNA analysis, isolation, and DNA sequencing analysis.** For the analysis of DNA, agarose or polyacrylamide gel electrophoresis was done under standard conditions. Recovery of DNA from either agarose or polyacrylamide gels by the Geneclean II kit (BIO 101 Inc., La Jolla, CA, U.S.A.) or the standard method, respectively.

Both directions of deletion plasmids with pYM1 and pUC19 were constructed with a Deletion Kit (Takara Shuzo Co., Ltd., Kyoto, Japan). Both strands of DNA were sequenced completely with an Applied Biosystems model 373A sequencer. The nucleotide and deduced amino acid sequences were analyzed with the GENETYX (Software Development Co., Ltd., Tokyo, Japan).

**Purification of extracellular protease.** The protein of a culture supernatant from B. subtilis MT-2 carrying pYM1 was precipitated with 60% saturated ammonium sulfate. The precipitate was dissolved in 10 mM Tris HCl buffer (pH 7.0), and the solution was then chromatographed on phenyl Toyopearl, DEAE Toyopearl and TSKgel G 2000sw HPLC columns. The enzyme fractions for neutral protease were collected and used for characterization of the protease.

**Protease assay.** Protease activity was routinely measured by a slight modification of a method of Burnett et al. A 100-μl sample of enzyme solution was incubated at 37°C in the presence of 1% azocaseine and 50 mM Tris hydrochloride (pH 7.5) in a total volume of 500-μl for 20 min. Reactions were stopped by adding 1.0 ml of 10% (w/v) trichloroacetic acid; precipitates were removed by centrifugation for 5 min at 15,000 rpm, and the absorbance of the supernatant fraction was measured.

We defined 1 unit of protease as the quantity required to increase the absorbance at 335 nm by the equivalent of 1 μg tyrosine min⁻¹ at 37°C.

**Results**

Cloning of protease production stimulating gene from B. steatorthermophilus HA19

The chromosomal DNA (about 5 μg) was digested with PstI and ligated with a PstI digest (about 1 μg) of pTBS1 in a total volume of 50 μl. The ligation mixture was used for transformation of B. subtilis MT-2 (npr, trpC2, leuC7, hsdR, hsdM1), and the transformants forming haloes on LC agar plates containing 5 μg ml of kanamycin were selected. About 10⁵ transformants were obtained, and of these, one colony could form a halo. The plasmid was isolated from the halo-forming one transformant on a LC agar plate, and was designated pYM1. The restriction map of pYM1 is shown in Fig. 1. The recombinant plasmid carried about a 2.2-kb fragment insertion from B. steatorthermophilus HA19 DNA.

![Fig. 1. Restriction Map of pYM1.](image)

Network bar indicates the 2.2-kb fragment from B. steatorthermophilus HA19. Replication origin and kanamycin resistance gene are identified by repA and Km', respectively. Numbers show the distance (kb) from PstI site.

![Fig. 2. SDS Polyacrylamide Gel Electrophoresis of the Culture Supernatants of B. subtilis MT-2 and MT-2 Carrying pYM1 (A), and Purified 37-kDa Protease from B. subtilis MT-2 Carrying pYM1.](image)

The arrows show the 37-kDa protease band. A: Lane 1, relative molecular mass standards (94.0-kDa, phosphorylase b; 67.0-kDa, ovalbumin; 30.0-kDa, carboxic anhydrase; 20.1-kDa, trypsin inhibitor); KDa, kilodaltons. Lane 2, culture supernatant from B. subtilis MT-2. Lane 3, culture supernatant from B. subtilis MT-2 carrying pYM1. B: Lane 1, purified 37-kDa protease. Lane 2, relative molecular mass standards (94.0-kDa, phosphorylase b; 67.0-kDa, ovalbumin; 30.0-kDa, carboxic anhydrase; 20.1-kDa, trypsin inhibitor); KDa, kilodaltons.
Protease expression by the cloned fragment

The protease expression by B. subtilis MT-2, neutral protease deficient strain, carrying pYM1 with L broth for 24 h of cultivation was analyzed by SDS-PAGE (Fig. 2A). The protease activity in the culture supernatant showed more than 2500 U per ml with azocaseine as a substrate.\(^5\)\(^-\)\(^7\)

About a 37,000 Da protein band was increased on the SDS–PAGE analysis (Fig. 2A). To check whether or not the cloned fragment from B. stearothermophilus HA19 stimulates protease production in another Bacillus strains, the pYM1 was also transformed into B. subtilis M1113 (neutral and alkaline protease producing strain) and B. subtilis DB104 (neutral and alkaline protease deficient strain), and the protease activities of both culture supernatants were measured. The protease activities were 3000 and 1800 U per ml with azocaseine as a substrate, respectively, and the about 37,000 Da protein band was also expressed on the SDS–PAGE from both culture supernatants (data not shown).

Purification and characterization of the protease

The protease was purified from 1 liter of culture supernatant from B. subtilis MT-2 carrying pYM1 by phenyl Toyopearl and DEAE Toyopearl opened column chromatography, and TSKgel G 2000sw HPLC column chromatography (Tosoh Corp., Tokyo, Japan). The purified fraction was analyzed by SDS–PAGE (Fig. 2B). The molecular weight (MW) was estimated as 37,000 by the SDS–PAGE analysis and gel filtration (TSKgel G 2000sw). The profile of the protease activity was investigated using a purified sample. The optimum pH of the protease were around pH 7 (Fig. 3). The enzyme was stable at pH 7–10 after 24 h at room temperature (Fig. 4). The optimum temperature of the enzyme activity was 50 °C (Fig. 5). The enzyme was inhibited by EDTA but not phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP) (Table I). These results indicate that the expressed protease B. subtilis is a neutral (metal) protease.

DNA sequence of the 2.2-kb fragment

The 2.2-kb PstI fragment from pYM1 was subcloned into the PstI site of pUC19, and the opposite-direction subclonal plasmids were designated pYMT1 and pYMT2, respectively. Several deletion plasmids were constructed for

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Fig. 4. pH Stability of the Stimulated Protease from B. subtilis MT-2. Enzyme solution was kept at room temperature for 24 h at each pH indicated. Then the solution was neutralized and the enzyme activity was measured at 37 °C.

Fig. 5. Optimum Temperature of the Stimulated Protease from B. subtilis MT-2. Enzyme activity was measured in 10 mM Tris HCl buffer (pH 7.5) at the temperatures indicated.

<p>| Table 1. Protease Inhibition with Inhibitors |</p>
<table>
<thead>
<tr>
<th>Inhibitor (^4) (mm)</th>
<th>Protease activity (%)</th>
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<tbody>
<tr>
<td>None</td>
<td>100</td>
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<tr>
<td>EDTA (1)</td>
<td>5</td>
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<tr>
<td>(5)</td>
<td>2</td>
</tr>
<tr>
<td>(10)</td>
<td>1</td>
</tr>
<tr>
<td>DFP (1)</td>
<td>92</td>
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<tr>
<td>(5)</td>
<td>93</td>
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<td>(10)</td>
<td>87</td>
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<td>PMSP (1)</td>
<td>97</td>
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<tr>
<td>(5)</td>
<td>90</td>
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<td>(10)</td>
<td>90</td>
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\(^4\) EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl fluorophosphate; PMSF, phenylmethylsulfonyl fluoride.
Fig. 6. Nucleotide Sequence of the 2.3-kb Protease Production Activator Gene.

The translated polypeptide is shown below the nucleotide sequence, with the one-letter designation of each amino acid residue positioned below the second nucleotide of the codon. Putative ribosome-binding sites are underlined and indicated (rbs). Start codons are preceded by the name of the ORF, and stop codons are indicated by asterisks.
DNA sequencing with a Takara deletion kit (Takara Shuzo Corp., Kyoto, Japan). The nucleotide sequence of the 2.2-kb fragment was analyzed by the dideoxy method (Fig. 6). The sequence had two open reading frames; one was 870 nucleotides (290 amino acid residues), and the other was 516 nucleotides (172 amino acids residues). The nucleotide and deduced amino acids sequences were analyzed with the GENETYX software. A search of the DNA database found 100% identity between the translated sequences of the cloned genes and previously described cell shape determinant genes from B. subtilis, mreC and mreD (Fig. 6).4,5

Construction of mreC or mreD deletion plasmids and the protease production carrying each plasmid

To identify the gene(s) stimulating the 37-kDa protease production in B. subtilis, we constructed mreC or mreD deletion plasmids (Fig. 7). The plasmid, pYM1, was digested with EcoRV, and the larger fragment was isolated and ligated. The resultant plasmid, pYM2, carries the mreC gene-deleting fragment. pYM3, the mreD gene-deleting plasmid, was constructed as follows. pYM1 was digested with SnaB1, and the PstI linker (dpGCTGCAAGC) was inserted into the site. The resultant plasmid, pYM3, had an introduced frame shift mutation downstream from the SnaB1 site in the mreD gene. The plasmids, pTB51, pYM1, pYM2, and pYM3, were used to transform B. subtilis MT-2, and the protease activities of those carrying these plasmids were measured (Table II). The strain MT-2 carrying pYM2 or pYM3 had as low protease activity as that carrying pTB51 (vector), indicating that the 37-kDa protease production in B. subtilis requires both mreC and mreD genes.

Discussion

The mreC and D genes were cloned and sequenced as cell shape determination genes from Escherichia coli and B. subtilis.3, 5, 16, 17 In E. coli, the genes of the mre operon (mreB, C, and D) are known to be involved in determination of cell shape, since mutation or deletion of the mre genes leads to formation of spherical cells.3,17 The loss of mreB, C, or D gene function also makes the cells become spherical.5, 18 However, the functions of MreC and MreD are unknown.18, 19 We found out here that the thermophilic bacteria B. steaorthermophilus has also mreC and D genes that are exactly the same as B. subtilis ones, and the genes stimulated the neutral protease production in Bacilli.

It is well known that Bacillus species produce several intra and extra-cellular proteases, and some alkaline proteases participate in spore formation in Bacilli.20–23 B. subtilis MT-2 and DB104 are major extracellular protease(s)-deficient strains but these strains are normal in cell shape formation, suggesting that these deficient major extracellular proteases in B. subtilis don’t participate in the cell shape determination. In this paper, the mreC and D genes, which were originally isolated from B. steaorthermophilus HA19, were shown to stimulate the 37-kDa protease production in B. subtilis. The optimum pH and temperature of the protease were 7.0 and 50 C, respectively. The protease was inactivated by EDTA. These results indicate that the 37-kDa protease is a neutral (metal) protease. The protease activities of B. subtilis MT-2 carrying the mreC or mreD deletion plasmids were at almost equal levels as that carrying the vector plasmid pTB51, indicating that the 37-kDa protease production in B. subtilis requires both mreC and mreD genes.

The cell shape determinant genes, mreC and mreD, must be located on the chromosomal DNA in B. subtilis MT-2 and DB104 because the strains form normal cell shapes. We show the stimulation of 37-kDa protease in B. subtilis carrying mreC and mreD genes on the vector plasmid pTB51, of which the copy number is 8 in B. subtilis.81 Since the mreC and mreD products are increased by the gene dosage effect in the host strains, the minor protease, of which expression is at a very low level in the normal condition in B. subtilis, may be stimulated by the increase in copies of MreC and MreD. The stimulated protease by mreC, D genes may be one of the minor proteases in B. subtilis, and related to the cell shape determination in Bacilli. We expect that the 37-kDa protease may be most
important cell shape determinant in *Bacilli*.

On the other hand, since the cloned *mreC, D* genes are
from a thermophilic *Bacillus*, we also expect that the same
mechanisms for shape determination should exist in ther-
mophilic *Bacillus*. Further work is needed to identify of the
role of *mreC* and *mreD* products in *B. subtilis* and *B.
steaurotherrnophilus*.

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