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Cloning and Strong Expression of a *Bacillus subtilis* WL-3 Mannanase Gene in *B. subtilis*

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Abstract A gene encoding the mannanase of *Bacillus subtilis* WL-3, which had been isolated from Korean soybean paste, was cloned into *Escherichia coli* and the nucleotide sequence of a 2.7-kb DNA fragment containing the mannanase gene was subsequently determined. The mannanase gene, designated *manA*, consisted of 1,080 nucleotides encoding a polypeptide of 360 amino acid residues. The deduced amino acid sequence was highly homologous to those of mannanases belonging to glycosyl hydrolase family 26. The *manA* gene was strongly expressed in *B. subtilis* 168 by cloning the gene downstream of a strong *B. subtilis* promoter of plasmid pJ27Δ88U. In flask cultures, the production of mannanase by recombinant *B. subtilis* 168 reached maximum levels of 300 units/ml and 450 units/ml in LB medium and LB medium containing 0.3% locust bean gum, respectively. Based on the zymogram of the mannanase, it was found that the mannanase produced by recombinant *B. subtilis* could be maintained stably without proteolytic degradation during the culture time.

Keywords: *Bacillus subtilis* WL-3, mannanase, nucleotide sequence, strong expression, recombinant *B. subtilis*

Mannan-type polysaccharides are some of the major constituents of the hemicellulose fractions of hardwoods and softwoods, as well as in the endosperm of many leguminous seeds and in some mature seeds of non-leguminous plants. Mannan materials include mannan, glucomannan, galactomannan, and galactoglucomannan, which consist of a β-1,4-linked linear backbone of mannose residues that carry other carbohydrates or acid substitutions. In general, there are three known enzymes, *i.e.*, endo-1,4-β-mannanase (mannanase), exo-1,4-β-mannanase, and β-mannosidase, that participate in the complete decomposition and conversion of mannan.

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Mannanases, which catalyze the random hydrolysis of the β-D-1,4-mannopyranosyl linkages within the backbone of various mannan-based polysaccharides, are useful enzymes within the food, feed, paper [33], and laundry industries. Because lots of grains are currently used as carbon sources for the microbial production of bioenergy, the prices of feed grains have escalated. Hence, much attention has been paid to feed additive enzymes [5, 12, 20, 23, 29]. Although the mannanases are widely distributed in microorganisms, higher plants, and animals, the microbial mannanases are regarded to be useful for their industrial applications. So far, numerous mannanases and their respective genes have been identified from bacteria and fungi. Bacterial mannanases have been characterized from various strains in 20 different genuses. These mannanases have been shown to belong to either glycosyl hydrolase (GH) family 5 or 26 on the basis of amino acid similarity. Although the mannanase hydrolyzes mannan polysaccharides that have a higher degree of polymerization than mannotetraose, which has a mannose polymerization degree of 4, some of the mannanases have been found to hydrolyze mannotriose [2, 18] or mannotetraose [10, 32].

The most important activity of mannanase, which is included as a hemicellulase together with xylanase and glucanase, is the saccharifying of hemicellulose, a recyclable plant resource, into a carbon source that is available to living organisms. Galactomannans of leguminous seeds are regarded as antinutritional factors, because monogastric animals are unable to digest them rapidly enough to obtain the edible sugars. Therefore, mannanase has been used as a feed additive enzyme to increase the nutritional value of soybean meal, a major component of animal feed [35]. For the application of mannanase in the fields of food processing and animal feed additives, we have previously isolated two mannanase-producing bacteria, *Bacillus subtilis* WL-3 [25] and WL-7 [16], from Korean soybean paste. In addition, two *Bacillus* strains producing valuable hydrolytic enzymes were isolated from fermented soybean [6, 26].

The mannanase genes were characterized from many strains of genus *Bacillus* including *Bacillus* sp. 5H [14], *Bacillus* sp. NM-39 [22], *Bacillus* sp. AM-001 [1], *Bacillus* sp. N16-5 [21], alkaline *Bacillus* sp. JAMB-750 [11], *B. subtilis* WL-7 [19], *B. circulans* [34], and *B. stearothemophilus* [7]. The present work describes the characterization of a WL-3 mannanase gene and the strong expression of the gene in *B. subtilis* 168.

MATERIALS AND METHODS

Chemicals and Enzymes

Restriction endonucleases, proteinase K, and RNase were obtained from Boehringer Mannheim (Mannheim, Germany). T4 DNA ligase was purchased from Solgent Co. (Daejeon, Republic of Korea). All of the enzymes were used according to the recommendations of the manufacturers. Locust bean gum (LBG) and dinitrosalicylic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bacterial medium was obtained from Difco (Detroit, MI, U.S.A.).

Bacterial Strains, Plasmids, and Media

B. subtilis WL-3 was used as the source of the gene coding for mannanase. *Escherichia coli* XL-1 blue (*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *relA*1 *thi lac*$^-$ F'[*proAB*$^+$ *lacI*^{q} *lacZ* M15 Tn10(*tet*^{r})]) was used as a host for recombinant plasmids. *B. subtilis* 168 was used as a host cell for the expression of the mannanase gene. The pUC19 plasmid was used for all cloning and sequencing experiments in *E. coli*, and plasmid pJ2788U was used as an expression vector in *B. subtilis*. *E. coli* and *B. subtilis* were cultured at 37°C in LB broth (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, per liter, pH 7.0). Antibiotics used for the selection of transformants were ampicillin (50 μg/ml) for *E. coli* and kanamycin (10 μg/ml) for *B. subtilis*.

DNA Manipulation and Construction of a *B. subtilis* WL-3 Genomic Library

The standard procedures of Sambrook *et al.* [28] were used for DNA manipulation. The chromosomal DNA was isolated from *B. subtilis* WL-3 cells that were grown exponentially in LB medium. The chromosomal DNA was completely digested with PstI, and DNA fragments ranging from 2 to 10 kb were prepared from agarose gel. The PstI-generated chromosomal DNA fragments were introduced into the dephosphorylated PstI site of pUC19. The ligation mixture was transformed into *E. coli* XL-1 blue by the electroporation method. The transformation of *B. subtilis* 168 cells was performed by the competence method.

DNA Sequencing and Computer Analysis

Restriction endonuclease-generated DNA fragments of the *B. subtilis* DNA were subcloned into pUC19. The nucleotide sequences of the fragments were determined with a DNA sequencer (ABI Prism 377, Perkin Elmer Co., Foster City, CA, U.S.A.). DNA and protein sequences were analyzed using the DNASIS (Hitachi Software Engineering, Japan) program.

Enzyme Assays

Mannanase activity was determined by measuring the amount of reducing sugars liberated during the hydrolysis of LBG by the dinitrosalicylic acid method [24]. The standard assay reaction mixture consisted of 0.5% (w/v) of the polysaccharide substrates supplemented with 50 mM sodium citrate buffer (pH 6.0) and enzyme to make a final volume of 0.3 ml. The reaction mixture was incubated at 50°C for 15 min. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per min.

Zymogram Analysis

Samples were loaded on a 10% SDS-polyacrylamide gel for electrophoresis. The protein samples including 12 mM Tris·Cl (pH 6.8), 5.0% glycerol, 0.4% SDS, 2.8 mM 2-mercaptoethanol, and 0.2% bromophenol blue were boiled at 95°C for 5 min and loaded on 10% gel. During electrophoresis, an agar replica that included 1.0% agarose, 0.1% konjac, and 50 mM sodium citrate (pH 6.0) was prepared. After electrophoresis, the gel was washed with 25% isopropanol and 50 mM sodium citrate (pH 6.0) to remove the SDS, and was washed several times with 50 mM sodium citrate (pH 6.0). The gel was then placed on the agar replica and incubated at 50°C for 30 min. The agar replica was soaked in 0.2% Congo red solution for 1 h and washed with 1 M NaCl solution for 1 h at room temperature. The clear zones of the mannanase activity were visualized by soaking the gel in 0.5% acetic acid solution.

RESULTS AND DISCUSSION

Cloning and Nucleotide Sequence of a *B. subtilis* WL-3 Mannanase

The genomic library of *B. subtilis* WL-3 was constructed in *E. coli* XL-1 Blue using pUC19 as a cloning vector. Approximately 3,000 transformants were transferred onto LB agar plates containing ampicillin. After overnight incubation, these plates were overlayed with soft agar (0.7%) containing 0.1% konjac glucomannan and 80 ppm trypan blue for screening the *E. coli* clone showing mannanase activity. After incubation for 2 h at 50°C, two colonies capable of hydrolyzing konjac glucomannan were obtained by scoring the clear zones around them. These colonies exhibited β-mannanase activity on konjac glucomannan and LBG galactomannan as substrates in the plate assay. Two recombinant plasmids, designated pM3C1

and pM3C2, were isolated from these *E. coli* clones and analyzed with various restriction enzymes. Restriction analysis indicated that the clones harbored an identical 7.0-kb insertion fragment, of which the orientation was found to be opposite in the two individual recombinant plasmids (data not shown). The cloned gene was designated as *manA* and the gene product was named MANA. To investigate the secretion of the mannanase produced by the *E. coli* clone containing the *B. subtilis* WL-3 mannanase gene, mannanase activity was measured using LBG as a substrate with the culture filtrate and cell-free extract of the *E. coli* clone grown in LB broth for 15 h. The mannanase activity (approximately 8 U/ml) of culture supernatant was comparable to that of cell-free extract, which indicated that the *B. subtilis* WL-3 mannanase was secreted in *E. coli* cells. It was reported that mannanase was equally localized in the extracellular and intracellular fractions of recombinant *E. coli* cells harboring the *B. subtilis* WL-7 mannanase gene [19]. However, Khanongnuch *et al.* [14] reported that the mannanase could not be secreted in *E. coli* cells transformed with a *Bacillus* sp. 5H mannanase gene.

The partial 2,679-bp sequence of the 7.0-kb insert on the recombinant plasmid pM3C1 was determined, and the deduced amino acid sequence of mannanase yielded an open reading frame (*orf*) of 1,080 nucleotides coding for a 360 amino acid protein with an estimated molecular mass of 40,266 Da (Fig. 1). The *manA* gene is preceded by *orf*1 (nt positions 139–1,080) and is followed by *orf*2 (nt positions 2,263–2,679). The *orf*1 encodes a 314 amino acid protein showing 64% identity to the phosphomannaose isomerase (PMI) of *B. halodurans* (GenBank Accession No. BAB07635) and *orf*2 encodes the amino termini of a truncated protein showing 90% homology to a 137 amino acid stretch of a major catalase in the spore of *B. subtilis* 168 (Accession No. NP_391742) (data not shown). The transcriptional directions of these genes were identical on the chromosomal DNA of *B. subtilis* WL-3. Three structural genes coding for phosphomannose isomerase, mannanase,

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GCGGCTGAATCAATATATTGATCTGCCCGACATTGAAAACCGCATCGTTCCCCCTCAATTAGGCATTCACTCCGGAGCGGCCGGTTCGCTCGTATTGGCG  100
GAGCAGGCCTTAGAAGCGGCAAACCGGGAGGCGGGAAAATGACGAAACTCTTATTTTTGGAACCGGTTTTTAAAGAAAGATTATGGGGCGGGACGAAGCT  200
GCGCGATACATTCGGGTATGAGATTCCTTCTGAGCGGACGGGAGAGTGCTGGGCGATTTCAGCTCATCCGCACGGCACATCCATCGTGCGGAACGGGCCT  300
TTTTCCGGAACATCATTACATCGTCTCTGGAATGAACACCCTGACCTGTTCGGACATCCGAAAGGAGACGCCTTTCCCCTGCTCACAAAAATTCTCGACG  400
CAAATATGGACTTATCCGTACAGGTTCATCCCGATGACGCATATGCACATCGGCATGAAAACGGAGAACTCGGAAAAACGGAATGCTGGTACATATTGGA  500
CTGCAAAAAAGACGCGGAGCTCATTTTAGGGCACCGCGCCCAAACGAGAGAAGACTTCGTTCGGCACATTGAACGAAACGAGTGGGAACACCTGCTCCGC  600
CGCATCCCCATTAAACCCGGCGACTTTTTCTATGTCCCGAGCGGCACACTGCACGCTTTATGTGAAGGTACGCTCGTTCTTGAAATCCAGCAAAGCTCTG  700
ATGCCACCTACCGGCTTTACGACTATGACCGGACGGATGGGAACGGGAATAAACGCGAGCTTCATCTTCAGAAAGCAATTGACGTAACAGGCATTCCGCA  800
TACCGATGAAAATGTAAAAAGCACAAAGCATCGGATCGGAAATGCTCTTATTACAACACTTGCCGAAACACCTTTCTTTTCAGTTTATCAATGGGATGTG  900
AGCGGGAAAGCATCATTCCCCGCACCCGGCCGGTATTTACTCGCAAGCGTCATTAAAGGACGCGGAGAACTGGATGGATGCCCCATTCACAAAGGCGATC 1000
ACTTTATCGTGCCGGCGGATTCCGGAGATTTTGTTATAGAAGGAGATTGTGAGGTTATCGTTTCACATCCTTCATCATCTTAAAAATCAGGAGGTCTTTT 1100
ATATGCTCAAAAAGTTAGCAGTCTGCCTGTCTATCGTTTTATTACTCTTAGGAGCCGCCAGTCCGATATCGGCTCACACCGTTTATCCCGTCAACCCAAA 1200
  M  L  K  K  L  A  V  C  L  S  I  V  L  L  L  L  G  A  A  S  P  I  S  A  H  T  V  Y  P  V  N  P  N
TGCCCAGCAGACGACAAAAGATATCATGAACTGGCTGGCGCACCTGCCCAACCGTTCAGAAAACAGGGTCATGTCCGGTGCGTTCGGCGGGTACAGCGAT 1300
  A  Q  Q  T  T  K  D  I  M  N  W  L  A  H  L  P  N  R  S  E  N  R  V  M  S  G  A  F  G  G  Y  S  D
GTCACTTTTTCAATGACAGAGGAAAACCGCTTGAAAAACGCGACGGGACAGTCTCCCGCCATCTACGGCTGTGACTATGGGAGAGGATGGCTGGAAACAG 1400
  V  T  F  S  M  T  E  E  N  R  L  K  N  A  T  G  Q  S  P  A  I  Y  G  C  D  Y  G  R  G  W  L  E  T
CGGATATCACCGATACTATCGATTACAGCTGCAACAGCAGCTTGATCTCATACTGGAAAAGCGGCGGCCTCCCTCAGGTCAGCCTGCATCTCGCAAATCC 1500
A  D  I  T  D  T  I  D  Y  S  C  N  S  S  L  I  S  Y  W  K  S  G  G  L  P  Q  V  S  L  H  L  A  N  P
GGCTTTTCCATCCGGAAACTATAAAACGGCCATCTCAAACAGCCAGTACAAAAACATCCTGGACCCTTCAACTGTTGAAGGAAAACGGCTTGAGGCGCTG 1600
  A  F  P  S  G  N  Y  K  T  A  I  S  N  S  Q  Y  K  N  I  L  D  P  S  T  V  E  G  K  R  L  E  A  L
CTCAGCAAAATCGCCGACGGCCTTACTCAGCTGAAAAATCAAGGCGTCACCGTTCTGTTCAGGCCGCTTCACGAAATGAACGGCGAGTGGTTCTGGTGGG 1700
L  S  K  I  A  D  G  L  T  Q  L  K  N  Q  G  V  T  V  L  F  R  P  L  H  E  M  N  G  E  W  F  W  W
GGCTGACAGGCTACAACCAAAAAGACACGGAAAGAATCTCGCTGTACAAAGAGCTTTACAAGAAGATATACCGCTATATGACAGAGACAAGAGGATTGGA 1800
G  L  T  G  Y  N  Q  K  D  T  E  R  I  S  L  Y  K  E  L  Y  K  K  I  Y  R  Y  M  T  E  T  R  G  L  D
TAACCTTTTGTGGGTGTATTCGCCGGATGCCAACAGAGACTTTAAAACAGACTTCTACCCAGGCTCATCTTATGTGGATATTACCGGTCTGGACGCTTAC 1900
  N  L  L  W  V  Y  S  P  D  A  N  R  D  F  K  T  D  F  Y  P  G  S  S  Y  V  D  I  T  G  L  D  A  Y
TTCACTGATCCGTATGCGATATCAGGCTATGATGAAATGCTGTCTCTGAAAAAACCGTTTGCCTTTGCCGAAACCGGTCCGTCCGGCAATATCGGAAGCT 2000
F  T  D  P  Y  A  I  S  G  Y  D  E  M  L  S  L  K  K  P  F  A  F  A  E  T  G  P  S  G  N  I  G  S
TTGATTATGCTGCTTTTATCAATGCGATCAGGCAAAAGTATCCCGAGACCACGTACTTTTTGACATGGGATGAACAATTAAGTCCGGCGGCCAATCAAGG 2100
F  D  Y  A  A  F  I  N  A  I  R  Q  K  Y  P  E  T  T  Y  F  L  T  W  D  E  Q  L  S  P  A  A  N  Q  G
CGCGCAAAGCCTTTATCAAAACAGCTGGACACTGAACAAGGGCGAAATATGGAACGGCGGATCTTTGACGCCGATCGCGGAATAACCGCTTCTTTTGAAA 2200
  A  Q  S  L  Y  Q  N  S  W  T  L  N  K  G  E  I  W  N  G  G  S  L  T  P  I  A  E  *
ATCGGTTTTAAAAAACGGCTCTGCTGGGCATATTGTTACCGTTCAAAAAGGAGGGTTCAACAATGGAAAATAACAAAAACAGCGAAGAAACGCTTTCCAG 2300
TAAGACGACCGGTAAAGGCGAGTCAGAAGACACGCTGACAAACCGGCAGGGCCATCCGGTCACTGACAACCAGAATGTCAGGACAGTCGGCAATAGAGGA 2400
CCGACGACATTGGAGAATTATGATTTTTTGGAGAAAATCAGCCACTTTGACCGCGAACGGATTCCCGAGCGCGTCGTTCACGCGCGCGGCGCCGGCGCAC 2500
ACGGATACTTTGAGGCCTACGGAAGTTTTGGAGACGAGGAGATTTCTAAATACACAAGAGCGAAAGTGTTTCAAAAGAAAGGAACGAAAACTCCGGCGTT 2600
TGTGCGGTTTTCTACCGTCAACCACGGAAAGCATTCCCCGGAAACATTGCGTGATCCCCGGGGTTTTGCCGTTAAGCTT                         2679
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Fig. 1. Nucleotide sequence and the deduced amino acid sequence of the mannanase gene.

The amino acid deduced from the open reading frame is indicated with the one-letter code below the nucleotide sequence. The putative ribosome binding site (SD) is underlined. Italicized amino acids indicate the putative signal peptide of mannanase. The nucleotide sequences in the box are cleavage sites for NaeI, which was used to make the recombinant pMANWL3 plasmid shown in Fig. 3. The numbers at the end of each line correspond to the nucleotide positions. The nucleotide sequence has been deposited with GenBank under the accession number DQ167409.

and manganese-containing catalase were arranged adjacently on the chromosomal DNA of *B. subtilis* 168 (GenBank Accession No. Z99107) and *B. subtilis* WL-7 [19], indicating that the putative major catalase gene of the *B. subtilis* WL-3 spore is differently located on chromosomes from both of the *B. subtilis* strains.

A putative start codon, ATG, of *manA* is located at nucleotide position 1,103, which is separated by 19 nucleotides after the termination codon of the phosphomannose isomerase (*pmi*) gene, whereas the termination codon, TAA, is located at position 2,185. The putative initiation codon of *manA* was expected according to a putative ribosome-binding site (AGGAGG), which was followed by the codon at a spacing of 8 bp. The start codon of mannanase genes was predicted to be ATG from *Bacillus* sp. 5H [14], *B. subtilis* NM-39 [22], and *B. subtilis* HB002 (Accession No. AF324506), whereas two mannanase genes of both *B. subtilis* 168 and *B. subtilis* WL-7 were found to use TTG as the start codon. A possible transcriptional terminator consisting of a palindromic sequence was not found in the intervening sequences between *pmi*, *manA*, and putative catalase genes, suggesting that they may be transcribed to a polycistronic mRNA or their transcriptions may be terminated by a ρ transcriptional termination factor.

The pattern of codon usage in the *manA* gene resembles that of the highly expressed *B. subtilis* gene in amino acid residues such as Ile, His, Asn, Lys, Glu, and Arg, and differs from that of the WL-7 mannanase gene [9]. In addition, the *manA* has a total G/C content of 49.4%, whereas the G/C content is 59.2% at the third base of the codon. The preference of WL-3 *manA* for G or C residues at the third base of genetic codons was 20% higher than that of the mannanase gene from *B. subtilis* WL-7 [19].

Comparison of *B. subtilis* WL-3 Mannanase with Other Mannanases

When the deduced amino acid sequence of a WL-3 mannanase was compared with the sequences of other mannanases in the NCBI database using the BLAST search program [4], the mannanase showed high homology with mannanases from *Bacillus* strains belonging to glycosyl hydrolase family 26 as follows: *B. subtilis* NM-39 (identity: 99%), *B. subtilis* 168 (identity: 74%), *B. subtilis* Z-2 (identity: 74%), *Bacillus* sp. 5H (identity: 75%), and *B. subtilis* WL-7 (identity: 74%). Amino acid sequences of the mannanases from the above *Bacillus* species are aligned in Fig. 2. These mannanases had a catalytic module, whereas many GH26 mannanases from alkaliphilic *Bacillus* sp. JAMB-

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W3  MLKKLAVCLSIVLLLLGA--ASPISAHTVYPVNPNAQQTTKDIMNWLAHLPNRSENRVMSGAFGGYSDVTFSMTE  73
NM  ******************--************************************************************  73
BS  *F**THIS*L*IF**AS*VL*K**E****S**********TV*********T****L********HD****A*  75
5H  *V**YTIS*L*LF**AS*VL*K**E****S**********AV*********T****L********HD****A*  75
W7  *F**THIS*L*IF**AS*VL*K**E****S**********TV*********T****L********HD****A*  75
Z2  *F**THIS*L*LF**AS*VL*K**E****S**********TV*********T****L********HD****A*  75

W3  ENRLKNATGQSPAIYGCDYGRGWLETADITDTIDYSCNSSLISYWKSGGLPQVSLHLANPAFPSGNYKTAISNSQ  148
NM  ************************************C************************************  148
BS  AD*IRS*************A*******N*E*S**V***GD*M****N**I**I*********Q**HF**P*T*D*  150
5H  AD*IRS*A**********A*******N*E*S**V****D******N**I**I*********Q**HF****T*D*  150
W7  AD*IRS*************A*******N*E*S**V***GD*M****N**I**I*********Q**HF**P*T*D*  150
Z2  AD*IRS*************A*******N*E*S**V****D*M****N**I**I*********Q**HF**P*T*D*  150

W3  YKNILDPSTVEGKRLEALLSKIADGLTQLKNQGVTVLFRPLHEMNGEWFWWGLTGYNQKDTERISLYKELYKKIY  223
NM  ************************************************************N*************  223
BS  ******S**A*****N*M********QE*E****P*******************S*****N*******Q******  225
5H  **K***S********N*M********QE*E****P*******************S*****N*******Q******  225
W7  **K***S**A*****N*M********QE*E****P*******************S*****N*******Q******  225
Z2  **K***S**A*****N*M********QE*E****P*******************S*****N*******Q******  225

W3  RYMTETRGLDNLLWVYSPDANRDFKTDFYPGSSYVDITGLDAYFTDPYAISGYDEMLSLKKPFAFAETGPSGNIG  298
NM  ************************************************************************  298
BS  H***D*****H*I******************A*****V******Q*A*S*N***QLTA*N*****T*V**QTAN*  300
5H  H***D*****H*********************A*****V******Q*A*S*K***QLTA*N*****T*V**QTAN*  300
W7  H***D*****H*I******************A*****V******Q*A*S*N***QLTA*N*****T*V**QTAN*  300
Z2  H***D*****H*I******************A*****V******Q*A*S*N***QLTA*N*****T*V**QTAN*  300

W3  SFDYAAFINAIRQKYPETTYFLTWDEQLSPAANQGAQSLYQNSWTLNKGEIWNGGSLTPIAE              360
NM  ****************Q*************************************                      360
BS  ****SL*****K****K*I***A*NDEW*A*V*K**SA**HD***********D*****V*               362
5H  ****SL*****K****K*I***A*NDEW***V*K**SA**HD***********D*****V*               362
W7  ****SL*****K****K*I***A*NDEW***V*K**SA**HD***********D*****V*               362
Z2  ****SL*****K*R**K*I***A*NDEW***V*K**SA**HD***********D*****V*               362
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Fig. 2. Comparison of the *B. subtilis* WL-3 mannanase with others mannanases.

The amino acid sequences of six mannanases from *B. subtilis* WL-3 (W3), *B. subtilis* NM-39 (NM), *B. subtilis* 168 (BS), *Bacillus* sp. 5H (5H), *B. subtilis* WL-7 (W7), and *B. subtilis* Z-2 (Z2) are indicated by the one-letter code and have been aligned by introducing gaps (hyphens) to maximize similarities. Residues identical to the amino acid sequence of the WL-3 mannanase are indicated by asterisks in other sequences. Numbers at the end of each line correspond to the amino acid position in the protein.

750 [3], *Clostridium thermocellum* [15], *Caldicellulosiruptor* sp. Rt8.B4 [8], *Cellulomonas fimi* [30], and *Caldibacillus cellulovorans* [31] were composed of at least two modules with high molecular weight.

The 24-amino-acids stretch in the N-terminus of the predicted amino acid sequence has properties similar to typical signal peptides in *Bacillus* sp., consisting of positively charged amino acids followed by a hydrophobic amino acid stretch. Helix-breaking residues such as Pro and Gly occurred at the fourth and eighth residues before the cleavage site, respectively, which are identical to those of *B. subtilis* NM-39 [22], whereas mannanases of *Bacillus* strains, including *B. subtilis* WL-7 [19], *Bacillus* sp. 5H [14], *B. subtilis* 168 (Accession No. CAB12407), and *B. subtilis* Z-2 (Accession No. AAV84100), contain a signal peptide of 26 amino acid residues. All mature forms of their five mannanases consist of 336 amino acid residues, although the lengths of their signal peptides are not identical. When the N-terminal regions of their mannanases can be compared with the cleavage sites of other signal peptides, the amino acid sequences (I-S-A or I-E-A) for the cleavage sites are not identical to a typical signal peptidase processing site (A-X-A) [27]. Kweun *et al.* [17] reported that the WL-7 mannanase was not secreted by recombinant *E. coli* carrying the mannanase gene without an N-terminal signal peptide region.

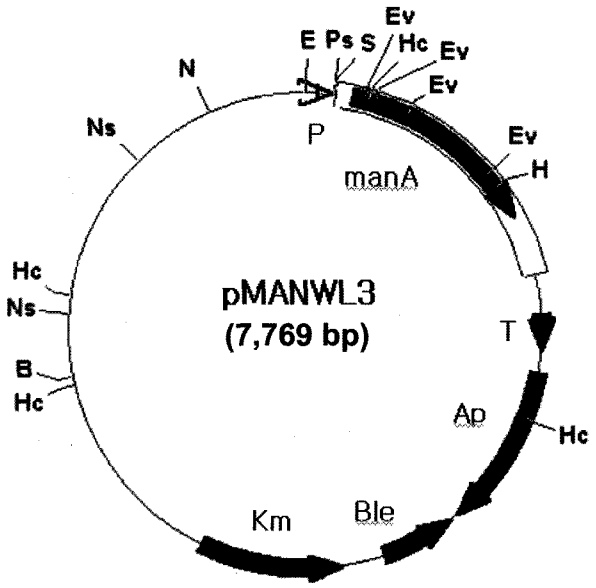

Fig. 3. Structure of expression plasmid pMANWL3 containing the mannanase gene.

The open bar indicates the NaeI fragment that includes a *B. subtilis* WL-3 *manA* gene, and the thin line indicates a region of expression vector pJ27Δ88U. P and T indicate the *Bacillus* promoter, BJ27Δ88, and the transcriptional terminator, $rrnBT_1T_2$, respectively. The structural genes for kanamycin resistance (Km), ampicillin resistance (Ap), bleomycin resistance (Ble), and mannanase (*manA*) are depicted. The arrows indicate the direction of transcription. Restriction site abbreviations are as follows: B, BglII; E, EcoRI; Ev, EcoRV; H, HindIII; Hc, HincII; N, NcoI; Ns, NsiI; Ps, PstI; S, SphI.

Strong Expression of the Mannanase Gene in *B. subtilis*

The previous report of the recombinant mannanase from *B. subtilis* WL-7 showed that the deletion of signal peptide sequences in the mannanase gene was favorable for strong expression of the enzyme in *E. coli* BL21(DE3) [17]. However, the mature mannanase was not secreted by recombinant *E. coli*. For strong expression of the *B. subtilis* WL-3 *manA* gene in *B. subtilis*, a recombinant plasmid containing the mannanase gene was constructed using pJ27Δ88U as an expression vector. It was previously reported that a *B. subtilis* CMCase gene was strongly expressed using this vector in *B. subtilis* [13]. The NaeI-generated 1.5-kb DNA fragment (nt 1,011–2491 of Fig. 1), which included the WL-3 *manA* gene, was introduced into the filled-in HindIII site of pJ27Δ88U containing a strong *Bacillus* promoter, BJ27Δ88, to create a recombinant plasmid, pMANWL3 (Fig. 3). It was found that the mannanase gene was located downstream from promoter BJ27Δ88 of pMANWL3. The plasmid was transformed into *B. subtilis* 168.

To estimate the mannanase productivity of *B. subtilis* (pMANWL3), the transformant was grown in LB medium for 18 h at 37°C. The mannanase activities of the culture filtrate were measured and compared with those of *B. subtilis* carrying a vector, pJ27Δ88U (Fig. 4). Because *B. subtilis* 168 has its own mannanase gene, *B. subtilis* 168 carrying pJ27Δ88U produced mannanase at 0.8 U/ml. The mannanase productivity of *B. subtilis* 168 (pMANWL3) reached 300 U/ml, which is approximately 375 times more than that of *B. subtilis* (pJ27Δ88U). In a previous work, the mannanase productivity of *B. subtilis* WL-3 in LB medium supplemented with LBG was found to be 131-fold more than that in LB medium [25]. To investigate whether the

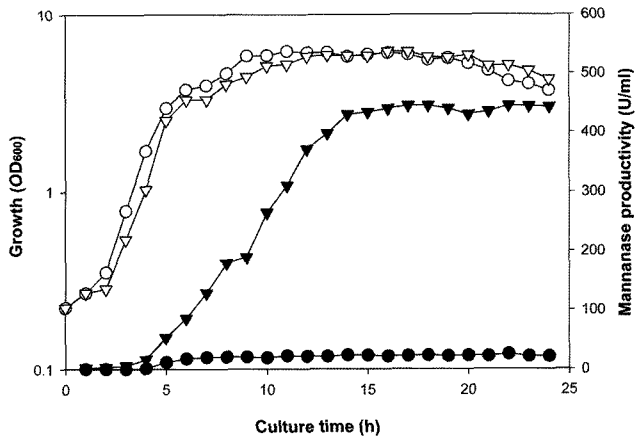

Fig. 4. Production of mannanase by the recombinant strains of *B. subtilis* 168 and their growths.

Growths (open symbols) and mannanase activities (closed symbols) in the culture filtrates of the *B. subtilis* carrying pJ27Δ88U (circle) and pMANWL3 (triangle) were observed periodically during growth in LB medium supplemented with LBG (0.3%) at 37°C, respectively.

increase of mannanase caused by the LBG occurs in recombinant *B. subtilis*, the recombinant *B. subtilis* was grown in LB medium supplemented with 0.3% LBG. The mannanase activity in the culture filtrate of *B. subtilis* (pMANWL3) reached a maximum level of 450 U/ml after cultivation for 15 h, whereas that of the *B. subtilis* (pJ27Δ88U) reached only 25 U/ml. The mannanase gene subcloned into pMANWL3 did not contain its own promoter, suggesting that the mannanase biosynthesis of *B. subtilis* (pMANWL3) was not induced by LBG. Therefore, the increase of mannanase production could be owing to utilization of LBG hydrolysates as carbon source by *B. subtilis* (pMANWL3). The cell growth patterns of the two strains were similar, which indicated that the strong expression of mannanase did not impede the growth of the host cells.

SDS-PAGE analysis of the culture filtrates of both *B. subtilis* (pJ27Δ88U) and *B. subtilis* (pMANWL3) showed that the secreted mannanase that had a molecular mass of approximately 38 kDa was dramatically increased by *B. subtilis* (pMANWL3) in relation to culture time (Fig. 5). However, mannanase showing weak activity was observed in the culture filtrate of *B. subtilis* (pJ27Δ88U). As a result of analyzing the plasmid DNA isolated from *B. subtilis* (pMANWL3), it was found that the recombinant pMANWL3 plasmid was stably maintained without structural instability, such as deletion or rearrangement on the plasmid, although the structural mannanase gene of WL-3 included a 684-nucleotides sequence that showed 73% identity to that of the *B. subtilis* 168 mannanase gene (*ydhT* in GenBank Accession No. Z99107).

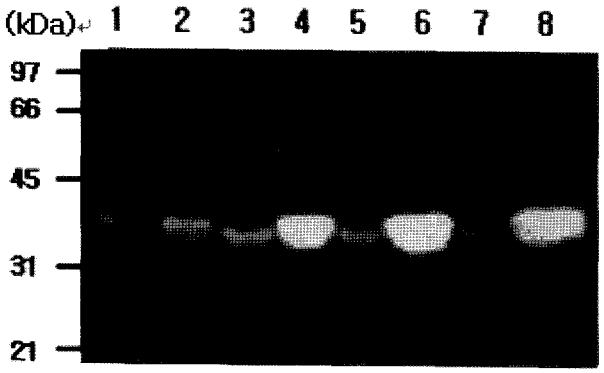

Fig. 5. Zymogram of the mannanase produced by recombinant *B. subtilis*.

B. subtilis 168 carrying pJ27Δ88U and *B. subtilis* 168 carrying pMANWL3 were grown for 12 h at 37°C in LB medium supplemented with LBG (0.3%). Culture filtrates were applied to 10% (w/v) SDS-polyacrylamide gel. Protein exhibiting mannanase activity was analyzed by activity straining. Lanes 1, 3, 5, and 7 indicate the 3 h, 6 h, 9 h, and 12 h cultures of *B. subtilis* (pJ27Δ88U) as a control, respectively. Lanes 2, 4, 6, and 8 indicate the 3 h, 6 h, 9 h, and 12 h cultures of *B. subtilis* (pMANWL3), respectively. Molecular size is shown in kilodaltons to the left side of the gel.

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