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Two *Bacillus* β-Mannanases Having Different COOH Termini Are Produced in *Escherichia coli* Carrying pMAH5

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The nucleotide sequence was determined for the alkalophilic *Bacillus* sp. strain AM-001 β-mannanase gene which produced two β-mannanases (A and B) in *Escherichia coli* transformants. The putative β-mannanase gene was 1,539 base pairs long and encoded a mature β-mannanase protein of 487 amino acids and a signal peptide of 26 amino acids. The COOH-terminal amino acid of β-mannanase A is an arginine residue located at amino acid 513 of the deduced amino acid sequence, and that of β-mannanase B is a valine residue located at amino acid 365. Deletion derivatives having 1,098 base pairs from the ATG start codon maintained the β-mannanase activity of the encoded polypeptide. However, clones harboring DNA fragments (1,051 base pairs) shorter than the gene which encoded β-mannanase B (1,095 base pairs) did not exhibit the β-mannanase activity. The simultaneous production of both β-mannanases A and B in an *E. coli* transformant was demonstrated by the maxicell procedure.

β-Mannanase (1,4-β-D-mannan mannanohydrolase; mannan endo-1,4-β-mannosidase; EC 3.2.1.78) is an enzyme that cleaves the β-1,4-mannosidic linkages of mannans, galactomannans, glucomannans, and galactoglucomannans. It is a potentially important enzyme for the utilization of various β-mannans present in some kinds of plants, such as endosperms of copra and ivory palm nuts, beans of guar, locust, and coffee, and roots of konjak.

Alkalophilic *Bacillus* sp. strain AM-001 grows well on various β-mannans as carbon sources (2) and produces significant amounts of three extracellular β-mannanases (3) and a cell-associated β-mannosidase (4). These three β-mannanases differ in their enzymatic properties, including optimum pH, optimum temperature, pH stability, thermal stability, isoelectric point, and molecular weight. To elucidate the genetic mechanism for the production of multiform β-mannanase by alkalophilic *Bacillus* sp. strain AM-001, we tried to clone the β-mannanase gene of this strain in *Escherichia coli*.

We recently constructed a hybrid plasmid, pMA1, which has a 4.0-kilobase (kb) DNA insert derived from the chromosome of alkalophilic *Bacillus* sp. strain AM-001 at the single *Hin*dIII site in the *lacZ'* gene of pUC19 (1). The insert was found to code for a β-mannanase gene expressible in *E. coli* transformants. A 2.0-kb *Xba*I-*Pst*I fragment (pMAH5) of the cloned DNA was found to be sufficient for β-mannanase synthesis in the subcloning study. We purified β-mannanase from the *E. coli* transformant (pMAH5), and two active fragments, β-mannanases A and B, were obtained. The molecular weights of purified β-mannanases A and B were estimated to be 58,000 and 43,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively. The cloned 2.0-kb DNA fragment has coding capacity for only one protein with a molecular weight of ca. 70,000 and insufficient coding capacity for these two enzymes unless overlapping genes exist in different reading frames.

To elucidate the mechanism for the synthesis of the β-mannanases synthesized in alkalophilic *Bacillus* sp. strain AM-001 and *E. coli*, we determined the DNA sequence of the β-mannanase gene. Here we present the nucleotide sequence of the β-mannanase gene, an analysis of the proteins produced from the cloned gene by the maxicell procedure, and the COOH-terminal amino acid sequences of the purified β-mannanases.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Alkalophilic *Bacillus* sp. strain AM-001, a producer of extracellular β-mannanases and a cell-associated β-mannosidase, was isolated in our laboratory (2). The other bacterial strains and plasmids used in this study are described in Table 1. LB medium (10) and x2TY medium (1) were used for liquid media, and LB agar medium containing 1% konjak mannan (16) was used for the plating medium.

Construction of a deletion mutant of the β-mannanase gene (*Xba*I-*Pst*I fragment). Recombinant plasmids containing a 2.0-kb chromosomal DNA insert from alkalophilic *Bacillus* sp. strain AM-001 were constructed to determine the nucleotide sequence of the β-mannanase gene and to locate the region encoding the β-mannanase B gene (Table 1). The 2.0-kb *Xba*I-*Pst*I fragment from pMAH5 was recloned on plasmid pUC118 (pMAH7) or pUC119 (pMAH8), respectively. Plasmid pMA1 was digested with *Xba*I and *Pst*I, and the resulting fragment (2.0 kb) was treated with the Klenow enzyme to fill in the termini. This fragment was inserted into the *Sma*I site of pHSG398. For construction of plasmid pMAU6, the *Eco*RI-*Hin*dIII fragment of pMAH6 was inserted into the *Eco*RI-*Hin*dIII site of pUC118. A series of deletion plasmids was constructed from pMAH8 and

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference
E. coli strains		
JM101	Δ(*lac-pro*) *thi supE* F′ *traD36 proAB lacI*^{q}ZΔM15	12
MV1184	*ara* Δ(*lac-pro*) *strA thi* (φ80 *lacZ*ΔM15) Δ(*sri-recA*)*306*::Tn*10* (Tet^r) F′ *traD36 proAB lacI*^{q}ZΔM15	19
CSR603	F$^-$ *recA1 uvrA6 phr-1 thi-1 thr-1 leuB6 lacY1 galK2 ara-14 xyl-15 mtl-1 proA2 argE3 rpsL31 tsx33 supE44 gyrA98* λ$^-$	13
Plasmids		
pHSG398	*E. coli* plasmid carrying Cm^r	17
pUC118	*E. coli* plasmid carrying Amp^r	19
pUC119	*E. coli* plasmid carrying Amp^r	19
pBR329	*E. coli* plasmid carrying Amp^r Tet^r Cm^r	5
pMAH5	*Xba*I-*Pst*I fragment and pHSG398 (at *Xba*I-*Pst*I site)	This paper
pMAU6	*Xba*I-*Pst*I fragment and pUC118 (at *Sma*I site)	This paper
pMAH6	*Xba*I-*Pst*I fragment and pUC118 (at *Xba*I-*Pst*I site)	This paper
pMAH7	*Xba*I-*Pst*I fragment and pUC119 (at *Xba*I-*Pst*I site)	This paper
pMAH8	*Xba*I-*Pst*I fragment and pHSG398 (at *Sma*I site)	This paper

pMAU6 as described by Henikoff (7) following treatment with a commercial Kilo-Sequence Deletion Kit obtained from Takara Shuzo Co., Ltd. (Kyoto, Japan) and a universal translation terminator (Pharmacia Fine Chemicals AB). Nucleotide sequence analysis was done to determine the deleted portion of the 3′ end for deletion plasmids. The deletion plasmids were transformed into *E. coli* MV1184.

Enzyme assay. *E. coli* strains harboring recombinant plasmids were analyzed for the presence of β-mannanase activity as follows. Overnight cultures of *E. coli* MV1184 harboring recombinant plasmids were grown in 2×TY medium containing 50 μg of ampicillin per ml at 37°C with shaking prior to harvesting. After sonication of the culture broth for 1 min, β-mannanase activity was assayed by the method reported previously (3).

Nucleotide sequencing. Nucleotide sequences were determined by the dideoxy chain termination procedure (15) with a commercial M13 Sequencing Kit obtained from Takara Shuzo Co. [α-^{32}P]dCTP was purchased from Amersham Japan. The nucleotide and amino acid sequences were analyzed with the GENIAS sequence analysis program (Mitsui Knowledge Industry Co., Ltd.).

Amino acid composition. Amino acid analysis was done with a Waters Associates, Inc., automatic PICO-TAG amino acid analysis system.

Amino acid sequence analysis. About 1 nmol of the purified peptide fragment was used for amino acid sequence analysis with an Applied Biosystems model 477A protein/peptide sequencer (6). Identification of phenylthiohydantoin derivatives of amino acids was performed in an Applied Biosystems model 120A phenylthiohydantoin analyzer.

COOH-terminal sequence analysis. Purified β-mannanase A was digested with tosyl lysine chloromethyl ketone–α-chymotrypsin (Sigma Chemical Co., St. Louis, Mo.), and purified β-mannanase B was digested with tolylsulfonyl phenylalanine chloromethyl ketone–β-trypsin (Sigma). The digested solutions were applied to an anhydrotrypsin agarose gel column (8), and the fractionated peptide fragments were analyzed for amino acid sequence and amino acid composition. COOH-terminal sequence analysis of purified enzymes was carried out at Toray Research Center, Inc. (Tokyo, Japan).

PAGE and immunoblotting. SDS-PAGE was done by the method of Laemmli (9). Stacking gels contained 4% acrylamide, and separating gels contained 12.5% acrylamide. After electrophoresis, the separated proteins were transferred electrophoretically to nitrocellulose sheets (Bio-Rad Laboratories, Richmond, Calif.) as described by Towbin et al. (18). For visualization of proteins cross-reactive with the antibody to β-mannanase, an immunological assay kit (Bio-Rad) was used. Preparation of antisera against β-mannanases A and B was carried out at Kohjin Co. (Tokyo, Japan).

Labeling maxicell proteins. Protein labeling was done by the maxicell procedure of Sancar et al. (14). *E. coli* CSR603 harboring plasmid pMAH5 was grown in M9 medium plus 1% Casamino Acids to an optical density at 600 nm of ca. 0.5 at 37°C and irradiated with UV light from a 15-W germicidal lamp (model GL-15; NEC Corp.) for 10 s. The irradiated culture medium was incubated at 37°C with shaking for 1 h, and D-cycloserine (final concentration, 100 μg/ml) was added. Incubation was continued at the same temperature overnight. Ten milliliters of the culture was centrifuged, and the collected cells were washed twice with Hershey salts. The washed cells were suspended in 5 ml of Hershey medium. After 1 h of starvation, L-[^{35}S]methionine (15.0 mCi/ml; Amersham Japan) was added to a final concentration of 5 μCi/ml, and incubation was continued for 5 to 60 min. The labeled cells were collected by centrifugation, suspended in 0.1 ml of lysis buffer (1% 2-mercaptoethanol, 1% SDS, 50 mM Tris [pH 6.8]), and heated for 3 min at 100°C. Ten-microliter samples were loaded onto a polyacrylamide-SDS gel as described above. After the run, the gel was dried and subjected to autoradiography for 1 to 3 days.

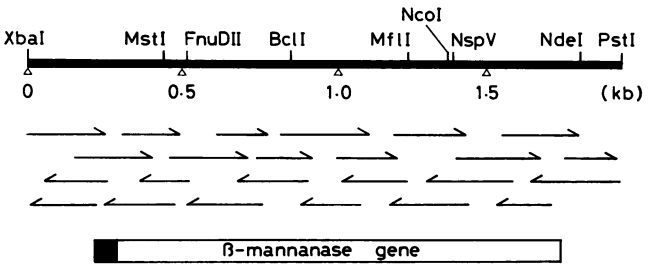

FIG. 1. Strategy for sequencing the β-mannanase gene of alkalophilic *Bacillus* sp. strain AM-001. A detailed restriction map of the *Xba*I-*Pst*I fragment encoding the β-mannanase gene and the sequencing strategy are shown. Arrows indicate the direction and extent of the sequencing reaction.

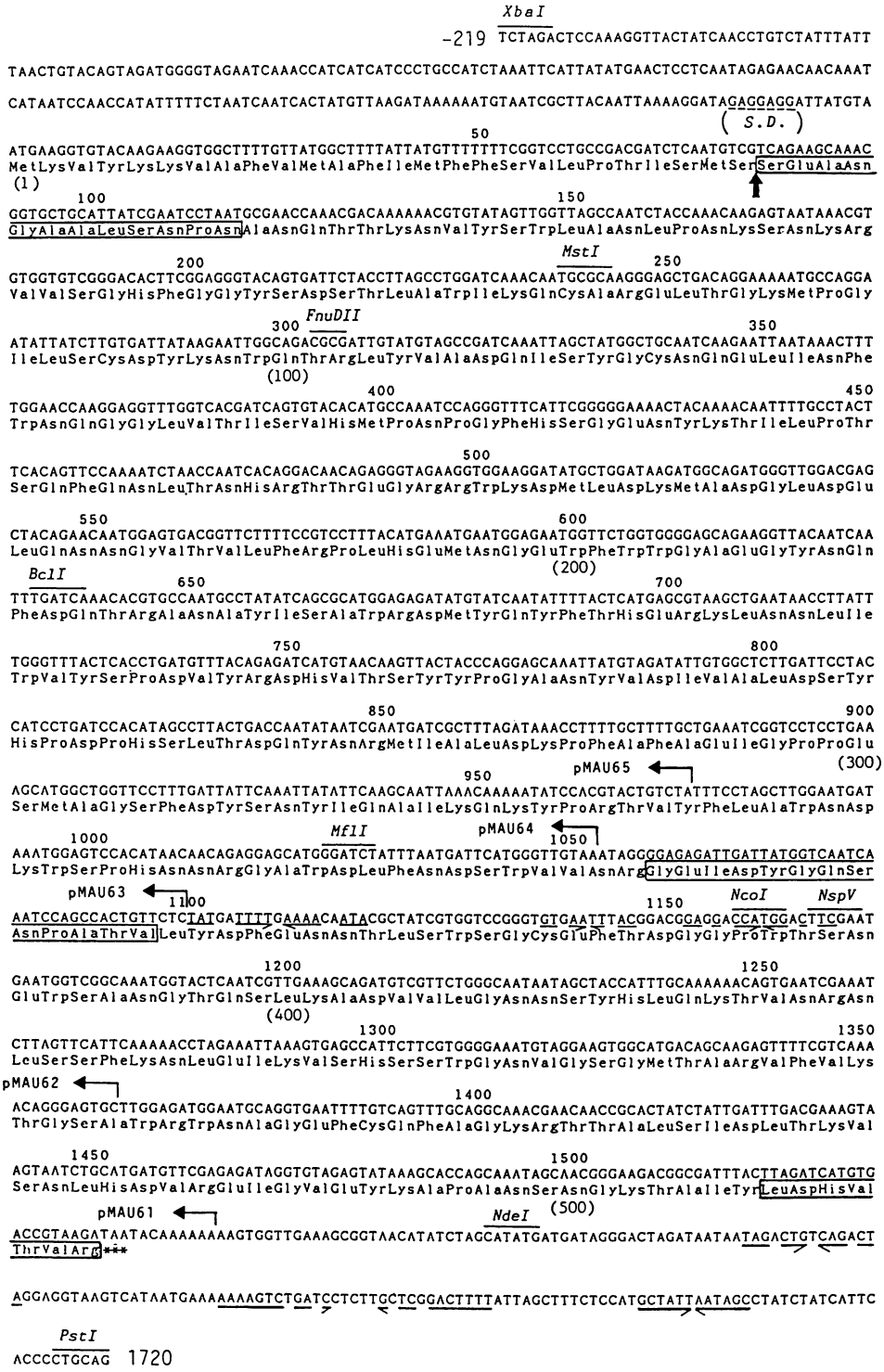

FIG. 2. Nucleotide sequence of the β-mannanase gene and the deduced amino acid sequence. The DNA sequence is given in the direction 5′ to 3′ and is numbered from nucleotide 1 at the putative initiation site. The proposed ribosome-binding site (Shine-Dalgarno, *S.D.*) is underlined with a dotted line. The predicted amino acid sequence is given below the DNA sequence. The deduced position of processing of the signal peptide is indicated by a vertical arrow. The boxed amino acids were determined by automated Edman sequencing of purified β-mannanases A and B. The sequences containing inverted repeat structures are designated by →←. The endpoints of deletion clones are represented by ←┐.

TABLE 2. Amino acid compositions of β-mannanases A and B as expressed in *E. coli* and as determined from the DNA sequence and by amino acid analysis

Amino acid	Amino acid composition of the following mannanase as determined by:			
	DNA sequence analysis[a]		Amino acid analysis[b]	
	A	B	A	B
Asx	73 (15.8)	53 (16.5)	16.0	16.7
Thr	30 (6.5)	18 (5.6)	6.3	5.8
Ser	39 (8.5)	23 (7.1)	8.4	6.7
Glx	39 (8.5)	29 (9.0)	8.0	8.6
Pro	19 (4.1)	17 (5.3)	4.8	5.2
Gly	36 (7.8)	23 (7.2)	8.8	7.4
Ala	33 (7.1)	23 (7.2)	7.4	7.0
Cys	5	3	ND	ND
Val	29 (6.3)	17 (5.0)	6.5	4.9
Met	9 (1.9)	8 (2.5)	1.7	2.5
Ile	18 (3.9)	14 (4.3)	3.5	4.2
Leu	34 (7.4)	23 (7.2)	7.8	7.3
Tyr	26 (5.6)	22 (6.9)	4.9	6.9
Phe	19 (4.1)	13 (4.1)	3.8	4.3
His	14 (3.0)	10 (3.1)	3.1	3.2
Lys	23 (5.0)	14 (4.3)	4.2	4.1
Arg	21 (4.5)	15 (4.7)	4.8	5.2
Trp	20	14	ND	ND

[a] Number of residues (moles percent).
[b] Moles percent. ND, Not determined.

RESULTS

COOH-terminal amino acid analysis. β-Mannanases A and B purified from an *E. coli* transformant (pMAH5) were used to determine the COOH-terminal amino acid sequence. The COOH-terminal sequence of β-mannanase A was determined up to residue seven to be Leu-Asp-His-Val-Thr-Val-Arg-COOH by amino acid sequencing. The COOH-terminal sequence of β-mannanase B was determined up to residue 13 to be Gly-Glu-Ile-Asp-Tyr-Gly-Gln-Ser-Asn-Pro-Ala-Thr-Val-COOH.

Nucleotide sequence. The complete nucleotide sequence of the 1,939-base-pair (bp) fragment harboring the β-mannanase gene was determined in both orientations (Fig. 1) and is shown in Fig. 2. An open reading frame containing 1,539 bp codes for the β-mannanase protein. The deduced amino acid sequence, which is encoded starting at the ATG codon beginning at position 1 and extending to termination codon TAA at position 1,539, contains 513 amino acids. A putative ribosome-binding-site sequence (GAGGAGG) is located eight nucleotides upstream from the translation initiation codon. The amino acid sequence from amino acids 27 (Ser, +1) to 38 (Asn, +12) coincides with the NH_2-terminal amino acid sequences of β-mannanases A and B obtained from the *E. coli* transformant (1). The cleavage site of the signal peptide and the mature protein is between the serine at amino acid 26 and the serine at amino acid 27. The amino acid sequences from amino acids 353 (Gly, +327) to 365 (Val, +339) and 507 (Leu, +481) to 513 (Arg, +487) coincide with the COOH-terminal amino acid sequences of β-mannanases B and A, respectively.

The calculated molecular weight of β-mannanase A, composed of 487 amino acid residues, was 55,430, and that of β-mannanase B, composed of 339 amino acid residues, was 39,085.

The amino acid compositions of β-mannanases A and B, calculated from the deduced amino acid sequence, approximately matched those determined chemically with purified enzymes from the *E. coli* transformant (Table 2). These results confirm that our DNA sequence data are essentially correct.

Deletion analysis of the β-mannanase gene on pMAH5. To determine which part of the protein is essential for β-mannanase activity, we sequentially deleted the *Xba*I-*Pst*I fragment from the 3′ end by using exonuclease III and mung bean nuclease. The remaining part of the gene was ligated to the 3′ end of *lacZ′* contained in pUC118, yielding plasmids pMAU61 through pMAU65 (Fig. 2 and 3). The deletion derivatives were transformed into *E. coli* MV1184, and the production of β-mannanase was tested. Of six clones, only clones 1 (pMAU6), 8 (pMAU61), 6 (pMAU62), and 23 (pMAU63) showed β-mannanase activity (Fig. 3). The other two clones did not show any enzyme activity.

The periplasmic fractions of the deletion mutants were tested with antisera prepared against β-mannanases A (data not shown) and B (Fig. 4). Clones 1, 8, 6, and 23 showed clear bands with both anti-β-mannanase A and anti-β-mannanase B in Western blotting (immunoblotting). Clones 6 and 23 showed three bands near the position of β-mannanase B. Clone 25 (pMAU64), which contained a 1,270-bp insert, did not show bands corresponding to β-mannanases A and B.

These results indicate that the minimum span for expression of β-mannanase activity is the region from position 1 to between positions 1,051 and 1,098 (Fig. 2).

Analysis of protein produced from the 2.0-kb *Xba*I-*Pst*I fragment. Proteins encoded within the *Xba*I-*Pst*I region were identified by analysis in the maxicell system. The *E.*

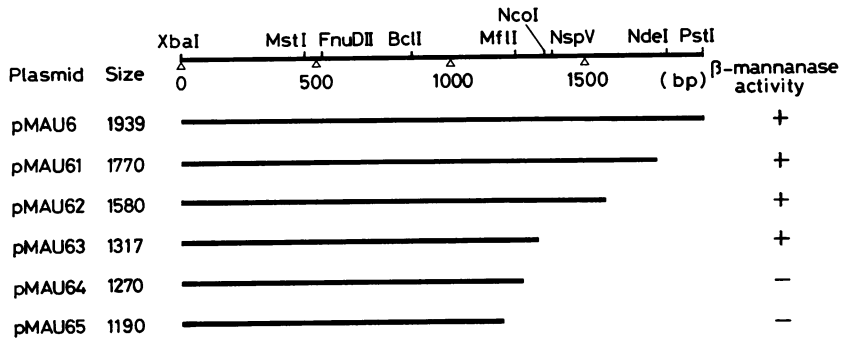

FIG. 3. Relationship between the structures of derivatives with deletions at the 3′ end of the β-mannanase gene and expression of β-mannanase in *E. coli* cells. Adjacent to each fragment is shown the ability of its product to exhibit β-mannanase activity. Sizes are in base pairs.

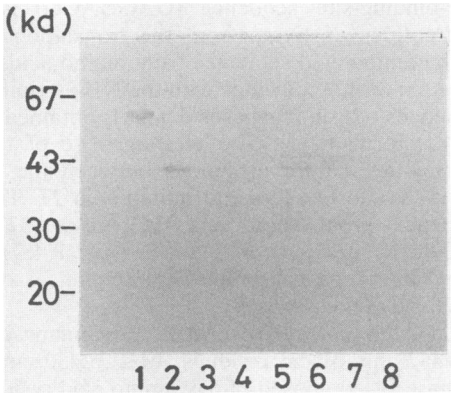

FIG. 4. Immunoblot analysis of proteins produced by various deletion mutants cross-reacting with anti-β-mannanase B sera. The molecular masses were estimated by SDS-PAGE with the following marker proteins (masses, in kilodaltons [kd], in parentheses): bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), and soybean trypsin inhibitor (20). Lanes: 1, β-mannanase A; 2, β-mannanase B; 3, periplasmic fraction of *E. coli* JM101, harboring pMAU6; 4, deletion clone 8 (pMAU61); 5, deletion clone 6 (pMAU62); 6, deletion clone 23 (pMAU63); 7, deletion clone 25 (pMAU64); 8, deletion clone 47 (pMAU65).

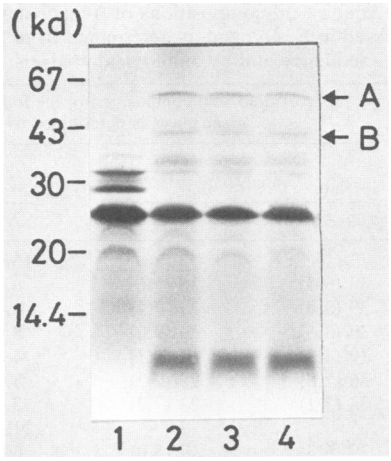

FIG. 5. Maxicell analysis of pMAH5-encoded proteins. The molecular masses were estimated by SDS-PAGE with the following marker proteins (masses, in kilodaltons [kd], in parentheses): bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), soybean trypsin inhibitor (20), and α-lactalbumin (14.4). Lanes: 1, vector control pBR329; 2, pMAH5 incubated with [^{35}S]methionine for 5 min; 3, pMAH5 incubated with [^{35}S]methionine for 30 min; 4, pMAH5 incubated with [^{35}S]methionine for 60 min. Arrows indicate β-mannanase A (A) and β-mannanase B (B).

coli culture containing pMAH5 was pulse-labeled with L-[^{35}S]methionine for various times, and the products were analyzed by SDS-PAGE and autoradiography. Vector gene products conferring resistance to ampicillin, tetracycline, and chloramphenicol are indicated in Fig. 5. Two protein bands with molecular weights of about 58,000 and 43,000 were observed (Fig. 5, lanes 2 to 4). These proteins were not observed in maxicells containing the plasmid without the *Xba*I-*Pst*I fragment. The 58,000-molecular-weight protein is β-mannanase A, and the 43,000-molecular-weight protein is β-mannanase B. The intensity of the β-mannanase bands in the autoradiogram did not change substantially during incubation with L-[^{35}S]methionine.

DISCUSSION

Nucleotide sequence analysis of the 2-kb *Xba*I-*Pst*I fragment of alkalophilic *Bacillus* sp. strain AM-001 showed the presence of a single open reading frame which coded for both of the β-mannanase proteins. The deduced amino acid sequence of mature β-mannanase A indicated a molecular weight of 55,430, which agreed closely with the value estimated by SDS-PAGE (1). The deduced amino acid composition of β-mannanase A indicated that it was a highly hydrophilic protein, containing 11.9% basic amino acids, 9.7% acidic amino acids, and 41.3% polar amino acids. The sequence of the N-terminal 12 amino acids of β-mannanases A and B (1) was identical to that deduced from the DNA sequence (nucleotide positions 79 to 114; Fig. 2). This result suggested that the first 26 amino acid residues, from the initiator Met to the Ser at position 76 in the nucleotide sequence, represented a single peptide which was removed during secretion of the β-mannanases. The amino acid sequence of this peptide contained several positively charged amino acids near the N terminus, followed by a hydrophobic amino acid core and Ser at the C-terminal end. The cleavage sites between Ser and Ser and the surrounding residues are in accordance with the −3, −1 rule proposed by von Heijne (20). A Shine-Dalgarno sequence, GAGGAGG (11), which is highly complementary to the 3′ end of *Bacillus subtilis* 16S rRNA (13), was observed 8 bp upstream of the initiation codon. We assume that a promoter which works in *E. coli* exists upstream of the open reading frame (1); however, no clear promoterlike sequence was found in the *Xba*I-*Pst*I fragment.

The COOH-terminal amino acid sequences of β-mannanases A and B were determined up to residues 7 and 13, and these sequences were identical to the deduced sequence. The COOH-terminal amino acids of β-mannanases A and B were Arg (513, +487) and Val (365, +339), respectively. The COOH-terminal amino acid of β-mannanase A coincided with one located within the open reading frame obtained from the nucleotide sequence analysis.

Although a single termination codon (TAA, 1,540 bp) is present after the Arg in β-mannanase A, such a codon is absent after the Val in β-mannanase B.

The simultaneous production of β-mannanases A and B was observed in maxicell preparations of plasmids carrying the *Xba*I-*Pst*I fragment at an initial stage (Fig. 5). This result may suggest that the production of the two β-mannanases is not caused by proteolytic cleavage of one precursor enzyme protein.

The following phenomena might have some regulatory function in the synthesis and secretion of β-mannanase B. (i) Although a termination codon is absent after the COOH-terminal amino acid, an earlier termination of transcription may be caused by several inverted repeat sequences found between positions 1,100 and 1,170 (Fig. 2). (ii) There may be an insufficient supply of tRNA corresponding to codon CTC, coding for Leu. This codon is present only once in the open reading frame, after the Val of the COOH-terminal amino acid (Table 3).

As the deletions from the 3′ end of the β-mannanase gene (*Xba*I-*Pst*I fragment) were tested, it became clear that the minimum span for expression of β-mannanase activity is the region from positions 1 to between positions 1,051 and 1,098 (Fig. 2 and 3). The deletion mutant (clone 25) harboring a deletion plasmid which is 45 bp shorter than the coding

TABLE 3. Codon usage of the β-mannanase gene

Codon	Amino acid	No. of residues	Codon	Amino acid	No. of residues	Codon	Amino acid	No. of residues	Codon	Amino acid	No. of residues
TTT	Phe	16	TCT	Ser	4	TAT	Tyr	16	TGT	Cys	3
TTC	Phe	7	TCC	Ser	3	TAC	Tyr	11	TGC	Cys	2
TTA	Leu	8	TCA	Ser	8	TAA		0	TGA		0
TTG	Leu	7	TCG	Ser	10	TAG		0	TGG	Trp	20
CTT	Leu	5	CCT	Pro	8	CAT	His	11	CGT	Arg	5
CTC	Leu	1	CCC	Pro	0	CAC	His	3	CGC	Arg	0
CTA	Leu	8	CCA	Pro	11	CAA	Gln	15	CGA	Arg	5
CTG	Leu	6	CCG	Pro	1	CAG	Gln	4	CGG	Arg	0
ATT	Ile	11	ACT	Thr	7	AAT	Asn	32	AGT	Ser	10
ATC	Ile	6	ACC	Thr	4	AAC	Asn	14	AGC	Ser	7
ATA	Ile	3	ACA	Thr	11	AAA	Lys	17	AGA	Arg	7
ATG	Met	13	ACG	Thr	9	AAG	Lys	9	AGG	Arg	4
GTT	Val	9	GCT	Ala	10	GAT	Asp	24	GGT	Gly	10
GTC	Val	5	GCC	Ala	6	GAC	Asp	3	GGC	Gly	4
GTA	Val	9	GCA	Ala	17	GAA	Glu	13	GGA	Gly	15
GTG	Val	10	GCG	Ala	2	GAG	Glu	7	GGG	Gly	7

region of β-mannanase B had no activity. The COOH-terminal region of β-mannanase B is apparently very important for the expression of β-mannanase activity.

To further elucidate the mechanisms of expression of β-mannanases A and B synthesized from one gene in *E. coli*, analysis of mRNA from *E. coli* JM101 (pMAH5) by Northern blot analysis is now in progress.

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