Molecular cloning and nucleotide sequence of a gene for alkaline cellulase from *Bacillus* sp. KSM-635

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A gene for alkaline cellulase from the alkalophilic *Bacillus* sp. KSM-635 was cloned into the *Hind*III site of pBR322 and expressed in *Escherichia coli* HB101. Although the recombinant plasmid contained two *Hind*III inserts of 2.6 kb and 4.0 kb, the inserts were found to be contiguous in the *Bacillus* genome by hybridization analysis. Nucleotide sequences of a 2.4 kb region which was indispensable for the production of cellulase, and the flanking, 1.1 kb region, were determined. There was an open reading frame (ORF) of 2823 bp in the 3498 bp sequence determined, which encoded 941 amino acid residues. Two putative ribosome-binding sites and a σ^{43} -type, promoter-like sequence were found upstream from an initiation codon in the ORF. The deduced amino-terminal sequence resembles the signal peptide of extracellular proteins. A region of amino acids, 249 to 568, of the deduced amino acid sequence of the cellulase from this organism is homologous with those of alkaline and neutral enzymes of other micro-organisms, but nine amino acid residues were found to be conserved only in the alkaline enzymes.

Introduction

Members of the genus *Bacillus* produce many kinds of extracellular hydrolytic enzymes, including cellulases (Priest, 1977). The cellulolytic enzymes of *Bacillus* have recently been the focus of much attention because of their potential use in the bioconversion of agricultural wastes into useful products (Ryu & Mandels, 1980) and in laundry detergents (Kawai *et al.*, 1988; Ito *et al.*, 1989). For the latter purpose, the enzyme must act at high pH, because of the alkalinity of laundry detergents. Production of such alkaline cellulases has been reported for alkalophilic *Bacillus* strains (Horikoshi *et al.*, 1984; Fukumori *et al.*, 1985) and for neutrophilic *Bacillus* sp. KSM-522 (Kawai *et al.*, 1988).

Recently, alkalophilic *Bacillus* sp. KSM-635, isolated in this laboratory, was also found to produce an alkaline cellulase (endo-1,4- β -glucanase, 1,4- β -D-glucan 4-glucanohydrolase, EC 3.2.1.4) in quantity (Ito *et al.*, 1989). In a study of this alkaline cellulase, it was found that the molecular mass of the enzyme as judged by gel filtration, varied from 100 kDa to 600 kDa depending upon culture conditions. This variation may indicate that the cellulase forms complexes, as reported for several

other bacterial cellulases (Yoshikawa *et al.*, 1974; Ait *et al.*, 1979; Wood *et al.*, 1982; Horikoshi *et al.*, 1984; Langsford *et al.*, 1984; Schellhorn & Forsberg, 1984).

In order to characterize such multiple forms of the alkaline cellulase, further enzymic and genetic analyses have been carried out. This report describes the molecular cloning of a gene for alkaline cellulase from *Bacillus* sp. KSM-635 and the determination of its nucleotide sequence. Furthermore, a deduced amino acid sequence of the alkaline cellulase from this micro-organism is compared with those of other alkaline and neutral cellulases, with respect to their optimum pHs for enzymic activity.

Methods

Bacterial strains, plasmids and bacteriophages. *Bacillus* sp. KSM-635, isolated from a soil sample (Ito *et al.*, 1989), was used as the source of a gene for alkaline cellulase. *Escherichia coli* HB101 (F⁻ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44 leuB6 thi-1*) and *E. coli* JM109 (*recA1 Δ (lac-pro) endA1 gyrA96 thi-1 hsdR17 supE44 relA1 F' traD36 proAB lacI^q Z Δ M15*) were used as the hosts. Plasmid pBR322 and bacteriophages M13mp18 and M13mp19 were used for cloning and nucleotide sequencing.

Culture media. *E. coli* strains were grown in LB broth, YT broth or 2 $\times$ YT broth (Miller, 1972). MYG broth [1.5% meat extract (LAB-LEMC0 powder, Oxoid)/0.5% yeast extract (Difco)/0.1% KH₂PO₄/1.0% glucose/0.5% Na₂CO₃; by weight] was used for the propagation

Abbreviation: ORF, open reading frame.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M27420.

of *Bacillus* sp. KSM-635. Solidified media contained 1.5% or 0.8% (w/v) Bacto agar (Difco).

Preparation of DNA. *Bacillus* sp. KSM-635 was grown in MYG broth at 30 °C for 2 d, with shaking, and harvested by centrifugation. The chromosomal DNA was prepared as described by Saito & Miura (1963). Plasmid DNA and the replicative form of M13 bacteriophage DNA were isolated by the alkaline extraction procedure (Birnboim & Doly, 1979). Covalently closed circular DNA was purified by equilibrium density gradient centrifugation on CsCl/ethidium bromide.

Construction of recombinant plasmids. The chromosomal DNA from *Bacillus* sp. KSM-635 and pBR322 DNA were digested with *Hind*III and ligated by T₄ DNA ligase (Boehringer Mannheim) overnight at 16 °C. The ligated molecules were used for transformation of *E. coli* HB101 by the method of Mandels & Higa (1970).

Detection of cellulase-positive transformants. Transformed cells were selected by growth on LB agar plates that contained 50 µg ampicillin ml⁻¹ (Sigma) and carboxymethylcellulose (2.0%, w/v) at 37 °C. Production of cellulase by the drug-resistant colonies was visualized by staining the agar plates with Congo red (Teather & Wood, 1982).

Assay of cellulase activity. *E. coli* carrying the recombinant plasmid was grown on LB broth that contained ampicillin (50 µg ml⁻¹) at 37 °C for 24 h. A cell-free extract was prepared by sonication. Cellulase activity expressed in *E. coli* was assayed as described previously (Ito *et al.*, 1989).

Nick-translation and hybridization analysis of DNA digests. A ³²P-labelled DNA probe was prepared using a nick-translation kit (Takara Syuzo) and [α -³²P]dCTP (410 Ci mmol⁻¹, 15.2 TBq mmol⁻¹; Amersham; Rigby *et al.*, 1977) and then purified by gel filtration on Sephadex G-50. DNA digests, subjected to electrophoresis on agarose gel, were denatured by treatment with 0.2 M-NaOH/0.5 M-NaCl and then transferred electrically to a nylon membrane (Zeta-Probe Blotting Membrane, Bio-Rad). The DNA digests on the membrane were hybridized with the DNA probe, according to the manufacturer's directions, and the resulting radioactive hybrids were exposed to X-ray film (New RX, Fuji).

Nucleotide sequencing. The nucleotide sequence was determined by the dideoxy method of Sanger *et al.* (1977), using an M13 sequencing kit (Takara Syuzo) and [α -³²P]dCTP. The template DNA was prepared from M13mp18 or M13mp19 phage vector, as described by Messing (1983).

Preparation of antiserum. Crude alkaline cellulase from *Bacillus* sp. KSM-635 was prepared as described previously (Ito *et al.*, 1989). The preparation was purified by column chromatography on DEAE-Toyopearl 650S (Tosoh) and Bio-Gel A-0.5 m (Bio-Rad). The purified enzyme (1 mg) was mixed with an equal volume of incomplete adjuvant and then injected subcutaneously into a white rabbit at 2- to 3-week intervals. The antiserum formed was used for double-immunodiffusion analysis.

Results

Cloning of a gene for cellulase

After screening for ampicillin-resistant and cellulase-positive transformants of *E. coli* HB101, eight clones were selected. All plasmids in the clones had two or more inserts in the *Hind*III site of pBR322. The insert of 4.0 kb was observed in all the plasmids. Additionally, seven

plasmids had a 2.6 kb insert. The smallest plasmid, named pBC100, contained inserts of 4.0 kb and 2.6 kb as *Hind*III fragments, and its restriction map is shown in Fig. 1.

pBC100-encoded cellulase was active over a range of pH values from 5.0 to 11.0 and exhibited maximum activity at pH 9.5. The pH-activity curve of the enzyme coincided with that of the alkaline cellulase from *Bacillus* sp. KSM-635 (data not shown).

Double-immunodiffusion was done using the anti-serum prepared against the cellulase purified from *Bacillus* sp. KSM-635. The cellulase from *E. coli* HB101 carrying pBC100 gave a single precipitin line which fused with that for the authentic cellulase from *Bacillus* sp. KSM-635 (data not shown).

Hybridization analysis of *Hind*III inserts of pBC100

In order to confirm that the 4.0 kb DNA insert was directly adjacent to the 2.6 kb insert, hybridization analysis was done using a ³²P-labelled probe prepared from the 1.1 kb *Xba*I fragment in pBC100 (the open box in Fig. 1). As shown in Fig. 2, *Xba*I digests of *Bacillus* DNA contained a 1.1 kb fragment that hybridized with the labelled *Xba*I 1.1 kb probe. Both the 2.6 kb and 4.0 kb fragments in *Hind*III digests of the *Bacillus* DNA also hybridized with the probe. Thus, the 2.6 kb and 4.0 kb *Hind*III inserts of pBC100 were proved to be contiguous in the *Bacillus* genome.

Localization of the cellulase gene

Sub-cloning analysis was done to locate the cellulase gene in the *Hind*III inserts of pBC100. The 4.0 kb insert was sufficient for the production of cellulase, and it was successfully trimmed down by *Bal*31 nuclease to a 2.4 kb fragment (the thickest line in Fig. 1), without loss of production of the enzyme. However, the 2.4 kb fragment was too small to encode the *Bacillus* sp. KSM-635 cellulase with a molecular mass of 100 kDa or more (Ito *et al.*, 1989). This result proves that the 2.4 kb fragment encodes only the region of the *Bacillus* cellulase that is essential for its activity.

Nucleotide sequence analysis

The nucleotide sequence of the 2.4 kb fragment essential for expression of cellulase activity, and that of its flanking region (1.1 kb), in the 2.6 kb *Hind*III insert were determined. The 3498 bp nucleotide sequence that was determined is shown in Fig. 3. Only one open reading frame (ORF), encoding a protein of approximately 105 kDa, was identified in the sequence. The ORF began

Fig. 1. Restriction map of plasmid pBC100. The middle and thickest lines indicate cloned *Hind*III fragments from *Bacillus* sp. KSM-635. The thickest line indicates a region that is essential for expression of cellulase activity. The open box under the map shows the 1.1 kb *Xba*I fragment used for preparation of the 32 P-labelled DNA probe for the hybridization analysis of cloned fragments.

Fig. 2. Hybridization analysis of the *Hind*III inserts of pBC100. DNA from *Bacillus* sp. KSM-635, digested with *Eco*RI (lane 1), *Hind*III (lane 2) or *Xba*I (lane 3), was electrophoresed on a 0.8% agarose gel and then transferred onto a nylon membrane. The DNA digests on the membrane were hybridized with the 32 P-labelled 1.1 kb *Xba*I fragment of pBC100. (a) Results of agarose gel electrophoresis of digests of KSM-635 DNA. (b) Autoradiograms of digests of KSM-635 DNA hybridized with labelled *Xba*I probe.

at nucleotide 1 (ATG) in the 2.4 kb fragment and ended at nucleotide 2824 (TAA) in the 2.6 kb insert. A *Hind*III restriction site at the 3' end of the 2.4 kb fragment was observed at nucleotide 1747. Upstream from the initiation codon of the ORF, there were two putative ribosome-binding sites (SD1 and SD2), each of which was highly complementary to the 3' end of 16S ribosomal RNA (rRNA) from *Bacillus subtilis* (Hager & Rabinowitz, 1985). The SD1 site occurred at nucleotide -65 (AAGGAGG) and the SD2 site occurred at

nucleotide -10 (GGAGGT). The sequence from nucleotide -134 to -106 was similar to the consensus sequence of the σ^{43} -type promoter of *B. subtilis* (Moran *et al.*, 1982). This sequence consisted of TAGACG as the -35 region and TATTAT as the -10 region. A complete inverted-repeat sequence was found downstream from the stop codon of the ORF (nucleotides 2837 to 2861).

The deduced amino acid sequence (941 residues), shown under the nucleotide sequence in Fig. 3, contained a short hydrophilic region from amino acids 1 to 9, followed by a hydrophobic region from amino acids 10 to 29. The hydrophilic region was slightly basic because of the presence of the three lysine residues. The hydrophilic-hydrophobic sequence is similar to the signal peptide of extracellular proteins from the genus *Bacillus* (Mezes & Lampen, 1985).

Homology of the amino acid sequence to those of other glucanases

The deduced amino acid sequence of the alkaline cellulase from *Bacillus* sp. KSM-635 was compared with those of other cellulases (endo-1,4- β -glucanases). The sequence, from amino acids 229 to 941, had high homology (approximately 72%) with that of alkaline cellulase from *Bacillus* sp. strain 1139 (Fukumori *et al.*, 1986a). Additionally, the region of amino acids 249 to 568 of the cellulase from *Bacillus* sp. KSM-635 was also homologous (38–40%) with sequences of two alkaline cellulases from *Bacillus* sp. N-4 (Fukumori *et al.*, 1986b) and four neutral cellulases from three strains of *Bacillus subtilis* (MacKay *et al.*, 1986; Robson & Chambliss, 1987; Nakamura *et al.*, 1987) and from *Clostridium acetobutylicum* P262 (Zappe *et al.*, 1988). Alignment of these homologous amino acid sequences is shown in Fig. 4. In these regions, 90 amino acid residues of the cellulase from *Bacillus* sp. KSM-635 were found to correspond with those of all the cellulases mentioned above.

-604 GGCACCTGAGGAAGTAGCG AATCCTGTTCTTGATGCACATCCATACTTAACTTCTGGATTGGC
 -540 TTCATGAGTCGTGATGAAAACGGGAAGTGACCATTACATGGACTG TTTGCATTTAATTATTCGGCACTGATTAGCTGTGGCATTTCGCT
 -450 TCTGCTCTTTCTGGAATGAAGTACGGGGTCCCAAGACTTGTCACT GCCATTGCCGATCAGTTATTCCAAGATGATCGAGACGAGATTCTA
 -360 AAGGACTTCTTTGAGTATGATGAGAAGGAGTTTGTGCGAACTGG *DraI* CCTTTAAACGTCTAAATGAACATAATAGCGAAAGGGCTTAACCAA
 -270 AAATATGAATTGAACCCACATAAATTTGTGGGTTTTTATTAATCA *AsnI* AAAAAATGGTAAATAAACCTATTTTAACAATGCTTATAACCATTT
 -180 TTCTATTTATTGCATAAAAAAATCAGTAAAAAATTCATTTATAT GTAGACGTAAATTAACAAATATTATATATACGAAAGCGGT
 -90 *Nsp(7524)V* TTCGAAAAATAGAGGAAGGAGGAGAGTTTTAGTTTTTGTGTTT *SspI* *AsnI* *-35* *-10* GTTTATTGTAAGCGTTTACTATTAATACATTTCTGGGAGGTTATT
 SD1 SD2
 1 ATGAAAATAAAGCAAATTAACAATCTTTATCTTTGCTTTTAATC ATCACACTCATTATGTCCTATTTGTTCTATGGCTTCAGCAAAC
 M K I K Q I K Q S L S L L L I I T L I M S L F V P M A S A N
 91 ACAATGAGTCTAAGTCTAATGCATTTCTTTCTGATGTTAAA AAACTTCTTGGTCTTTTCCATATATAAGGATTTATATGAGCAA
 T N E S K S N A P P F S D V K K T S W S F P Y I K D L Y E Q
 181 GAAGTTATTACAGGAACATCTGCAACAACGTTCTCTCCAACAGAT TCCGTTACTCGTGACAATTTACAGTGATGCTTACCGTGGTCTT
 E V I T G T S A T T F S P T D S V T R A Q F T V M L T R G L
 271 GGACTAGAAGCATCTTCTAAAGATTACCCTTTTAAAGATCGTAAA *DraI* AACTGGGCTTACAAAGAAATTCAGCTGCATATGAAGCTGGAATT
 G L E A S S K D Y P F K D R K N W A Y K E I Q A A Y E A G I
 361 GTAAGTGGGAAAAACAAACGGTGAATTTGCACCAATGAAAACATT ACTCGTGAACAAATGGCTGCTATGGCCGTACGTGCTTATGAATAC
 V T G K T N G E F A P N E N I T R E Q M A A M A V R A Y E Y
 451 TTAGAAAATGAGCTATCTTTACCAGAAGAGCAAAGAGAATATAAT GACTCTTCTTCTATTTCAACCTTTGCTCAAGATGCTGTTCAAAAA
 L E N E L S L P E E Q R E Y N D S S S I S T F A Q D A V Q K
 541 *SnaBI* GCATACGTATTAGAGCTAATGGAAGGAAATACAGATGGATATTT CAACCAAAAAGAACTCTACTAGAGAACAGTCTGCTAAAGTTATC
 A Y V L E L M E G N T D G Y F Q P K R N S T R E Q S A K V I
 631 TCTACTTTACTTTGGAAAGTAGCTAGTCATGATTATTTATACCAT ACAGAAGCTGTTAAAGCCCTTCAGAAGCTGGTGCCTTCAGTTA
 S T L L W K V A S H D Y L Y H T E A V K S P S E A G A L Q L
 721 GTAGAATAAAGGACAATTAACACTAGCTGGTGAAGATGGTACT CCCGTTCAATTACGTGGAATGAGTACACATGGCCTACAATGGTTC
 V E L N G Q L T L A G E D G T P V Q L R G M S T H G L Q W F
 811 GGTGAAATCGTAAACGAAAACGCTTTCTAGCACTATCGAATGAT TGGGGATCTAACATGATTCTGCTCGCTATGTACATTGGCGAAAAT
 G E I V N E N A F V A L S N D W G S N M I R L A M Y I G E N
 901 GGATATGCAACAAACCTGAAGTAAAGATTTAGTTTATGAAGGA ATTGAATTAGCGTTTGAGCACGATATGTATGTAATTGTTGACTGG
 G Y A T N P E V K D L V Y E G I E L A F E H D M Y V I V D W
 991 CATGTACATGCTCCTGGTGATCCTAGAGCGGATGTATACTCAGGT *Nsp(7524)V* GCTTATGATTTCTCGAAGAAATGCTGATCATTACAAAGATCAT
 H V H A P G D P R A D V Y S G A Y D F F E E I A D H Y K D H
 1081 CCGAAAAACCATTTATCATTTGGGAACTAGCAAACGAACCAAGT CCAAATAATAACGGTGGACCTGGATTAACAAATGATGAAAAAGGT
 P K N H Y I I W E L A N E P S P N N N G G P G L T N D E K G
 1171 TGGGAAGCTGTAAAGAATATGCAGAGCCAAATCGTTGAAATGTTG CGTGAAAAAGGTGACAACATGATTTTAGTTGGAAATCCTAAGTGG
 W E A V K E Y A E P I V E M L R E K G D N M I L V G N P N W
 1261 AGCCAACGCTCTGACTTATCAGCTGACAACCAATTGATGCAGAA AATATCATGTATTCTGTTCACTTCTACACAGGCTCACATGGCGCT
 S Q R P D L S A D N P I D A E N I M Y S V H F Y T G S H G A

Fig. 3. Nucleotide sequence of the cellulase gene and deduced amino acid sequence. The nucleotide sequence of the coding strand is given from 5' to 3', and the deduced amino acid sequence is shown under the nucleotide sequence. Numbering of both nucleotides and amino acids starts with the beginning of the coding sequence. Putative -35 and -10 regions of the promoter are labelled with double dashed lines. Two putative ribosome-binding sites (SD1 and SD2) are underlined. Horizontal arrows indicate terminator-like, inverted-repeat sequences. A putative signal sequence is indicated by a broken line under the amino acid sequence. A vertical open arrow indicates the 3' end of the 2.4 kb fragment that is essential for expression of cellulase activity.

Fig. 4. Comparison of the amino acid sequence of alkaline cellulase from *Bacillus* sp. KSM-635 with those of alkaline and neutral enzymes from other micro-organisms. Amino acid residues conserved in all the cellulases are indicated by asterisks. Amino acid residues conserved only in alkaline cellulases or in neutral cellulases are boxed with bold or thin lines, respectively. The vertical arrow indicates the carboxy-terminus (Leu-584) of the truncated cellulase encoded by the 2.4 kb fragment. Ba, *Bacillus* sp. (alkalophilic); Bs, *Bacillus subtilis*; Ca, *Clostridium acetobutylicum*.

Furthermore, nine amino acid residues were found to be conserved only in the alkaline cellulases, and 18 amino acid residues were found to be conserved specifically in the neutral cellulases in these homologous regions.

Discussion

A gene for alkaline cellulase from *Bacillus* sp. KSM-635 was cloned and its nucleotide sequence was determined. In the 3498 bp sequence there was only one ORF, beginning with an ATG initiation codon (nucleotide 1) and ending with a TAA stop codon (nucleotide 2824).

Upstream from the initiation codon of the ORF, two putative ribosome-binding sites were found, one beginning at nucleotide -65, AAGGAGG (SD1), and another beginning at nucleotide -10, GGAGGT (SD2). Free energies of binding (ΔG) of SD1 and SD2 were calculated to be $-17.8 \text{ kcal mol}^{-1}$ ($1 \text{ kcal} \approx 4.2 \text{ kJ}$) and $-16.6 \text{ kcal mol}^{-1}$, respectively (Tinoco *et al.*, 1973), if they were to bind to the 3' end of the 16S rRNA from *B. subtilis*. The SD1 sequence was more highly complementary to the 16S rRNA, but it was 68 nucleotides away from the ATG initiation codon of the ORF. Immediately downstream from the SD1, there was no ATG, TTG or

GTG codon, any of which would be a plausible initiation codon in such a bacterium (Gold *et al.*, 1981). The ΔG value of the SD2 sequence and the distance of five nucleotides between the SD2 and the ATG are in reasonable agreement with data for other ribosome-binding sites reported for *Bacillus* strains (Hager & Rabinowitz, 1985). Hence, the SD2-ATG sequence is the probable site of initiation of transcription of the cellulase gene in *Bacillus* sp. KSM-635.

The sequences TAGACG and TATTAT, found at nucleotides -134 to -106, were highly homologous to the -35 region and -10 region, respectively, of the σ^{43} -type vegetative promoter of *B. subtilis* (Moran *et al.*, 1982). There were 17 nucleotides between these sequences, with an AT-rich region, which is common to the σ^{43} -type promoter. In a preliminary experiment, the *AsnI*-*Nsp*(7524)V (nucleotides -233 to -90) fragment (Fig. 3) has been cloned into the promoter-selection vector pKK232-8 (Brosius, 1984), which encodes the structural gene for chloramphenicol acetyltransferase. *E. coli* HB101 transformed by pKK232-8 containing the *AsnI*-*Nsp*(7524)V fragment acquired resistance to chloramphenicol. A σ^{43} -type promoter sequence immediately upstream from the site of initiation of transcription of the endo-1,4- β -glucanase gene of *B. subtilis* DLG (Robson &

Chambliss, 1987) has been found. Consensus-like sequences of the σ^{43} -type promoter have also been reported for some cellulase genes from *B. subtilis* (MacKay *et al.*, 1986; Nakamura *et al.*, 1987; Seo *et al.*, 1986). With respect to genes for alkaline cellulase in alkalophilic strains of *Bacillus*, no definitive sequence that might serve as a promoter of transcription has so far been observed.

The inverted-repeat sequence from nucleotide 2837 to 2861 had a calculated ΔG of $-13.6 \text{ kcal mol}^{-1}$ for the stem-loop structure. There was neither a poly(T) segment immediately downstream from the stem-loop structure nor a GC-rich segment in the stem, both of which are characteristics of a ρ -independent terminator (Adhya & Gottesman, 1978). Recently, the ρ factor that regulates the termination of transcription was found in *B. subtilis* (Hwang & Doi, 1980). The inverted-repeat sequence may act as a ρ -dependent terminator of transcription of the cellulase gene in *Bacillus* sp. KSM-635. Another inverted-repeat sequence ($\Delta G = -14.0 \text{ kcal mol}^{-1}$) with a GC-rich segment and an adjacent, short, T-rich segment was observed upstream from the promoter region of the cellulase gene (from nucleotide -258 to -245); it may be a ρ -independent terminator of an unidentified gene.

From the nucleotide sequence determined, an amino acid sequence of the alkaline cellulase from *Bacillus* sp. KSM-635 was deduced. The complete gene encoded the enzyme of 941 amino acid residues with a calculated molecular mass of 104626 Da. However, a carboxy-terminal sequence of 357 amino acid residues seems not to be necessary for the enzymic activity, because the 2.4 kb fragment essential for expression of the activity does not encode this region.

The sequence from amino acids 249 to 578, which was encoded in the 2.4 kb fragment, had high homology with sequences of three alkaline cellulases (Fukumori *et al.*, 1986a, b) and four neutral cellulases (MacKay *et al.*, 1986; Robson & Chambliss, 1987; Nakamura *et al.*, 1987; Zappe *et al.*, 1988) from strains of *Bacillus* and *Clostridium acetobutylicum* P262. When the homologous sequences were suitably aligned, 90 amino acid residues were found to be conserved in all the cellulases. Some of these conserved amino acid residues may play an important role in the expression of activity of the cellulases. In particular, tryptophan residues showed a remarkably high degree of conservation, i.e. 10 of 10 or 11 tryptophan residues, in the homologous region of each cellulase, were found to be conserved. Furthermore, nine amino acid residues were conserved only in the alkaline cellulases and 18 amino acid residues were conserved only in the neutral cellulases within the regions of high homology. Some of these residues may determine the optimum pH for activity of the cellulases. Such amino

acid residues, specific for alkaline or neutral cellulases, appear to be concentrated in the carboxy-terminal regions of the homologous amino acid sequences shown in Fig. 4. Specific amino acid residues, that were conserved only in alkaline cellulases from strains of *Bacillus*, have also been shown by Horikoshi & Fukumori (1988).

A putative signal sequence in the cellulase from *Bacillus* sp. KSM-635 consists of a hydrophilic region of seven amino acid residues, including three lysine residues, and a hydrophobic region of 22 amino acid residues. A short, hydrophilic, basic region and a subsequent, long, hydrophobic region are characteristic of *Bacillus* signal peptides (Murphy *et al.*, 1984; Mezes & Lampen, 1985). The residues Ala-Ser-Ala, amino acids 27-29, in the carboxy-terminal region of the hydrophobic region may be the site required for recognition by a signal peptidase (Perlman & Halvorson, 1983).

If the signal peptide is cleaved at the carboxy-terminal of residue Ala29, the molecular mass of the mature extracellular enzyme is calculated to be 101412 Da, a value close to that of the smaller cellulase observed in the culture broth of *Bacillus* sp. KSM-635 (Ito *et al.*, 1989). A larger enzyme, with a molecular mass of 300-600 kDa, as judged by gel filtration, was also observed in the culture broth, and it may represent aggregates of the smaller enzyme that is encoded by the gene identified in this study. In order to confirm a possible aggregated form of the alkaline cellulase, complete purification and characterization of the enzyme from *Bacillus* sp. KSM-635 are currently in progress in this laboratory.

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