Molecular cloning and nucleotide sequence of the gene encoding an endo-1,4- β -glucanase from *Bacillus* sp. KSM-330

KATSUYA OZAKI,* NOBUYUKI SUMITOMO and SUSUMU ITO

Tochigi Research Laboratories of Kao Corporation, 2606 Akabane, Ichikai, Haga, Tochigi 321-34, Japan

(Received 14 May 1991; revised 11 July 1991; accepted 22 July 1991)

The gene encoding an acid endo-1,4- β -glucanase from *Bacillus* sp. KSM-330 was cloned into the *Hind*III site of pBR322 and expressed in *Escherichia coli* HB101. The recombinant plasmid contained a 3.1 kb *Hind*III insert, 1.8 kb of which was sufficient for the expression of endoglucanase activity in *E. coli* HB101. Nucleotide sequencing of this region (1816 bp) revealed an open reading frame of 1389 bp. The protein deduced from this sequence was composed of 463 amino acids with an M_r of 51 882. The deduced amino acid sequence from amino acids 56 through 75 coincided with the amino-terminal sequence of the endoglucanase, Endo-K, purified from culture of *Bacillus* sp. KSM-330. The deduced amino acid sequence of Endo-K had 30% homology with that of the *celA* enzyme from *Clostridium thermocellum* NCIB 10682 and 25% homology with that of the enzyme from *Cellulomonas uda* CB4. However, the Endo-K protein exhibited no homology with respect to either the nucleotide or the amino acid sequences of other endoglucanases from *Bacillus* that had been previously characterized. These results indicate that the gene for Endo-K in *Bacillus* sp. KSM-330 has evolved from an ancestral gene distinct from that of other *Bacillus* endoglucanases.

Introduction

Cellulolytic enzymes have been reported in several strains of *Bacillus* (Tewari & Chahal, 1977; Dhillon *et al.*, 1985; Fukumori *et al.*, 1986*a, b*; MacKay *et al.*, 1986; Nakamura *et al.*, 1987; Robson & Chambliss, 1987; Au & Chan, 1987; Baird *et al.*, 1990). However, cellulases from *Bacillus* have received very limited attention, probably because most of these enzymes hydrolyse carboxymethylcellulose (CMC) but barely hydrolyse crystalline forms of cellulose and because they have had no commercial applications until recently.

We have isolated some strains of *Bacillus* that produce alkaline endo-1,4- β -glucanase (EC 3.2.1.4), the properties of which fulfil the essential requirements for enzymes to be used in laundry detergents (Okoshi *et al.*, 1990; Shikata *et al.*, 1990; Yoshimatsu *et al.*, 1990). A mutant strain of one of these isolates, *Bacillus* sp. KSM-635, is currently exploited for the industrial production of an alkaline endoglucanase for use in such detergents

(Yoshimatsu *et al.*, 1990). We have cloned and sequenced the gene for the endoglucanase from *Bacillus* sp. KSM-635 (Ozaki *et al.*, 1990). The deduced amino acid sequence of the alkaline endoglucanase protein exhibited significant homology to the amino acid sequences of other alkaline (Fukumori *et al.*, 1986*a, b*) and neutral (MacKay *et al.*, 1986; Nakamura *et al.*, 1987; Robson & Chambliss, 1987; Zappe *et al.*, 1988) endoglucanases produced by strains of *Bacillus* and by *Clostridium acetobutylicum*, with some amino acid residues conserved in either the alkaline enzymes or the neutral enzymes, or in both types of enzyme.

In order to clarify the mechanism of action of endo-1,4- β -glucanases, with a special focus on differences in pH optima, we have purified and characterized an acid endo-1,4- β -glucanase, Endo-K, produced by *Bacillus* sp. KSM-330 (Ozaki & Ito, 1991). Endo-K is active over an extremely narrow range of pH values, between 4.5 and 6.5, with an optimum pH at 5.2. One or more tryptophan residues in the enzyme molecule appear to be necessary for the expression of the endoglucanase activity. This report describes the cloning and sequencing of the gene encoding Endo-K to clarify the structure of the enzyme and to allow comparisons to be made between it and other alkaline or neutral endoglucanases.

Abbreviation: CMC, carboxymethylcellulose.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M68872.

Methods

Bacterial strains and cloning vectors. *Bacillus* sp. KSM-330 was used as the source of the gene for Endo-K. This micro-organism, a relative of *Bacillus subtilis*, was isolated from a soil sample (Ozaki & Ito, 1991). *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 Δlac-pro endA1 gyrA96 thi-1 hsdR17 supE44 relA1 F' traD36 proAB lacZ ΔM15*) were used as hosts for cloning and sequencing. Plasmid pBR322 and bacteriophages M13mp18 and M13mp19 were used as the vectors.

Media. *E. coli* strains harbouring plasmids were grown on LB broth or on M9CA medium (Maniatis *et al.*, 1982), each of which contained 50 µg ampicillin ml⁻¹ (sodium salt, Sigma). 2 × YT broth was used for cultivation of *E. coli* JM109 infected with M13 bacteriophage (Messing, 1983). P-medium contained (w/v) 1.0% Polypepton (Nihon Pharmaceutical), 0.5% yeast extract (Difco), 0.1% KH₂PO₄, 0.25% Na₂HPO₄·12H₂O and 0.02% MgSO₄·7H₂O, and was used for the propagation of *Bacillus* sp. KSM-330. Solid media contained 0.8 or 1.5% (w/v) Bacto agar (Difco).

Preparation of DNA. *Bacillus* sp. KSM-330 cells grown on P-medium at 30 °C for 24 h were harvested by centrifugation (9800 g, 10 min) at 4 °C. Genomic DNA was prepared from precipitated cells as described by Saito & Miura (1963). Plasmid DNA and the replicative form of M13 bacteriophage DNA were isolated by the alkaline extraction procedure of Birnboim & Doly (1979). Covalently closed circular DNA was purified by CsCl/ethidium bromide equilibrium density gradient centrifugation.

Cloning of the endoglucanase gene. Genomic DNA from *Bacillus* sp. KSM-330 and plasmid pBR322 DNA were both digested with *Hind*III (Boehringer Mannheim) and ligated with T4 DNA ligase (Boehringer Mannheim). The ligation mixture was used for transformation of competent *E. coli* HB101 cells (purchased from Takara Shuzo). Transformants that appeared on LB agar plates supplemented with ampicillin were overlaid with 1.0% (w/v) agar that contained 0.5% (w/v) CMC (degree of substitution 0.68, Sanyo Kokusaku Pulp), 1.0% (w/v) NaCl, 0.1 mg lysozyme ml⁻¹ and 50 mM-citric acid/sodium citrate buffer (pH 5.2). After incubation at 37 °C for 5 h, production of the endoglucanase activity by the transformants was detected by staining the overlaid CMC with Congo red dye, as described by Teather & Wood (1982).

Assay of endoglucanase activity. *E. coli* carrying the recombinant plasmid was grown on LB broth supplemented with ampicillin at 37 °C for 24 h. Cells were harvested from 35 ml of the culture by centrifugation (9800 g, 10 min) at 4 °C and were resuspended in 7 ml 10 mM-MOPS/NaOH buffer (pH 7.0). The suspension of cells was sonicated at 4 °C, and the cell debris was removed by centrifugation (12000 g, 15 min) at 4 °C. The endoglucanase activity of the supernatant was assayed as described previously (Ozaki & Ito, 1991). The preparation of enzyme was incubated at 40 °C for 60 min with 100 mM-citrate buffer (pH 5.2) that contained 1.0% (w/v) CMC (degree of substitution 0.68, Sanyo Kokusaku Pulp), and the amount of reducing sugar liberated was measured by the 3,5-dinitrosalicylic acid procedure (Miller *et al.*, 1960). One unit (U) of enzymic activity was defined as the amount of enzyme that produced 1.0 µmol of reducing sugar as glucose per minute under the conditions of the reaction.

Hybridization analysis of DNA digests. *Hind*III digests of *Bacillus* sp. KSM-330 genomic DNA (5 µg) were subjected to electrophoresis on a 0.8% (w/v) agarose gel and then electrophoretically transferred to a Zeta-Probe blotting membrane (Bio-Rad) after denaturation by soaking the gel with 0.2 M-NaOH/0.5 M-NaCl for 30 min at room temperature. Approximately 0.4 µg of the *Hind*III insert, isolated from the recombinant plasmid, was labelled with digoxigenin-11-dUTP and

used as a probe for hybridization analysis of the *Hind*III digests of the genomic DNA on the membrane, by use of a DNA-Labeling and Detection Kit (Boehringer Mannheim).

Nucleotide sequencing. Restriction fragments were subcloned into an appropriate position of the multiple cloning site of M13mp18 or M13mp19, and ordered deletion clones for sequencing were prepared by the method of Henikoff (1984), using a Kilo-Sequence Deletion Kit (Takara Shuzo). Single-stranded DNA was prepared by the procedure of Messing (1983). The nucleotide sequence was determined by the dideoxy chain-termination method of Sanger *et al.* (1977), using a fluorescent dye primer (Smith *et al.*, 1986) and a modified T7 DNA polymerase (Sequenase, United States Biochemical), on an automated DNA sequencer (Applied Biosystems). Approximately 300 bases were read from a sample in each lane, and both strands of the endoglucanase gene were completely sequenced.

Results and Discussion

Cloning of the gene for Endo-K

Among several thousand ampicillin-resistant transformants of *E. coli* HB101, one was found to produce endoglucanase activity, as detected by the procedure for staining with Congo red dye. This endoglucanase-expressing transformant contained a 7.7 kb recombinant plasmid (pKC3301) carrying two *Hind*III inserts of 3.1 and 0.2 kb in the *Hind*III site of pBR322 (Fig. 1). *E. coli* HB101 harbouring pKC3301 produced intracellular endoglucanase activity at a rate of approximately 10 U per litre of broth, when cells were grown at 37 °C for 24 h on LB broth that contained ampicillin. The plasmid-encoded endoglucanase was active from pH 4.2 to 7.0 with a maximum activity at pH 5.2, and it was active from 10 to 60 °C with a maximum activity at 45 °C (data not shown). These characteristics of the activity correspond to those of the Endo-K secreted by *Bacillus* sp. KSM-330 (Ozaki & Ito, 1991).

To clarify the origin of the 3.1 kb insert of pKC3301, hybridization analysis was done using the digoxigenin-labelled 3.1 kb insert of pKC3301 as a probe. The labelled insert of pKC3301 hybridized with a 3.1 kb fragment in *Hind*III digests of the genomic DNA from *Bacillus* sp. KSM-330 blotted on a membrane (data not shown). Thus, the 3.1 kb *Hind*III insert in pKC3301 appeared to have been derived from the genomic DNA of *Bacillus* sp. KSM-330.

Location of the gene for Endo-K

The 3.1 kb insert isolated from pKC3301 was subcloned into the *Hind*III site of pBR322 using *E. coli* HB101 as host strain. Two plasmids, pKC330 and pKC330R, were obtained, carrying the insert in opposite orientations. The yields of Endo-K activity of *E. coli* HB101 that

Fig. 1. Restriction map of plasmid pKC3301. The thick line indicates cloned *Hind*III fragments from genomic DNA of *Bacillus* sp. KSM-330. The stippled area indicates a region that is essential for expression of endoglucanase activity.

Table 1. *Endoglucanase activities isolated from E. coli HB101 cells carrying various plasmids*

Plasmid	Growth (OD ₆₀₀)	Activity [U (l culture) ⁻¹]
pKC3301	5.1	9.9
pKC330	5.6	13.4
pKC330R	5.0	12.1
pBR322	5.9	0

carried pKC330 and pKC330R were almost the same as or slightly higher than that of *E. coli* HB101(pKC3301), regardless of the orientation of the 3.1 kb insert (Table 1). Thus, it was confirmed that the gene for Endo-K was included in the 3.1 kb insert and that the gene was probably expressed under the control of its own promoter in this insert. In addition, the enzyme activity was detected in the recombinant *E. coli* HB101, even when a 1.0 kb *Hpa*I fragment of the 3.1 kb insert was deleted from pKC3301 by subcloning. This observation suggested that the gene for Endo-K is located in a 1.8 kb *Hind*III–*Hpa*I fragment, as shown by the stippled area in Fig. 1.

Nucleotide sequence analysis

The nucleotide sequence of the 1.8 kb *Hind*III–*Hpa*I fragment was determined. Only one large open reading frame (ORF), beginning with an ATG codon at nucleotide 226 and ending with a TAA codon at nucleotide 1615, was identified in the 1816 bp nucleotide sequence that was determined (Fig. 2). Upstream from this ORF, there is a putative ribosome-binding site with a

sequence of AAGGGAGATGA, followed five bases later by a potential initiation codon, ATG. The sequence would exhibit a free energy of binding (ΔG) of -13.0 kcal mol⁻¹ (-54.6 kJ mol⁻¹), as calculated by the method of Tinoco *et al.* (1973), if it bound to the 3' end of the 16S rRNA from *B. subtilis*. The ΔG value and the distance between the initiation codon and this putative ribosome-binding site agree reasonably well with data for other reported ribosome-binding sites from several strains of *Bacillus* (Hager & Rabinowitz, 1985).

The region upstream from the ORF was scanned for sequences with homologies to various types of promoter consensus sequence recognized by RNA polymerases from *B. subtilis* (Doi & Wang, 1986). The sequence from nucleotides 35 to 65 resembles the consensus sequence of the sigma A-type vegetative promoters of *B. subtilis* (Moran *et al.*, 1982). This sequence consists of TA-GAAA as the potential -35 region and TATATT as the potential -10 region, separated by 19 nucleotides. A sequence, resembling the consensus sequence of sigma C-type promoters of *B. subtilis* (Johnson *et al.*, 1983), was found upstream from the putative ribosome-binding site of the gene from *Bacillus* sp. KSM-330. This sequence begins at nucleotide 181; it includes the potential -35 region of AAATT and the potential -10 region of AATTTATTTTA; and the two regions are separated by 14 nucleotides.

A large inverted-repeat sequence was found downstream from the termination codon of the ORF (from nucleotides 1691 to 1732). The ΔG value of this sequence for a stem-loop structure was calculated to be -19.8 kcal mol⁻¹ (-82.9 kJ mol⁻¹), which could be sufficient for termination of transcription. However, there is neither a GC-rich segment in the stem nor a poly(T) segment downstream from the stem, both of which are considered to be characteristics of ρ -independent terminators of transcription in bacterial genes (Adhya & Gottesman, 1978). Nevertheless, the combination of potential promoter and terminator sequences suggests that the gene for Endo-K may be monocistronic.

Amino acid sequence analysis

The ORF in the nucleotide sequence encoded 463 amino acid residues, as indicated under the nucleotide sequence in Fig. 2. The molecular mass of the unprocessed protein was calculated to be 51882 Da. There was a short, relatively basic, hydrophilic region, from amino acids 1 to 6, followed by a hydrophobic region that extended from amino acids 7 through 30. This hydrophilic-hydrophobic sequence resembles the signal peptide of extracellular proteins of *Bacillus* (Mézes & Lampen, 1985). The deduced sequence from amino acids 56 to 75 is identical to the amino-terminal 20 amino acid residues

^{HpaI}
 GTTAACTATTTAAGTATTAACTACCATAGTTATAGAAATTCCT TAGATGAGTACTCTTATATTAGAAAAGCATAAAAATATACCAAG TCAGACTTCGTTTGGAAATATCTAACTGTATTCCTCAATCCTTCA
 45 90 135

 AAAAAAGGTACGTTTGTATGGAGTAAATATCATAGAAATACG AAATTTTATCTGAATTTGTAATTTTATTTAAGGGAGATGAAAA ATGTTAGAAAAAAGAAAGATATTCACCTGTTTTATGTGCATGTGGG
 180 225 270

 ATTGGGTTTACAAGCTATAGTTGTATTTACAGCAGCAGCTATA GACAATGATACATTAAATAATAATGACACAAAAATCAATCTTCT ATTATAACAAAACTCTTCTCAAGTATCGCTGCTGCTAAAGAGATG
 315 360 405

 AAACCTTTTCCCTCAACAAGTAAATTTACTCAGGAATTTTAAACCG AATCATGTTTTCACAGGAAGTTTGAATATGCAATGTTAAAAATAT TATAACGATTGGAAGAAGAAGTATCTAAAGAATGATTATCTTCA
 450 495 540

 CTTCTGCTGGGTTATGTTAAGGGTGAGATTACGGGAATCCCA GATGGGTTTAGACCATTAAGCACTTCAGAAGGACAAAGGATATGGG ATGATTATCACAGTTTTTAATGCGAGGACATGATTCAAATGCTCAG
 585 630 675

 ACTATTATGATGGCTTATTTAAACACAGCAAGAGCTTTTAAAGT TCCATAAACCCCAATTTAATGGGTGGGTTGTAGCAGATGACAAG AAAGCACAAGGGCATTTTGAATCTGCAACAGATGGGATTTAGAC
 720 765 810

 ATTGCTTATTCACCTCTTCTGTCATAGCAATGGGGATCAAGT GGGAAAAATCAATTTAATAAGAAAGCTCAAAATATGATTACAAAAA GGTATTAAGGCTAGTAATGTGACAAAAAATATGGTTTGAATTTA
 855 900 945

 GGGGATTGGGAGACAAAAAGTACATTTGATACCTAGACCATCCGAC TGGATGATGTCCTCATTTAAGGGCATTCACGAAATTTACAGGAGAT AAAACTTGGCTTAATGTTATTGATAATCTCTACATACTATATACA
 990 1035 1080

 AATTTACAAATAAATTCACCAAGACAGGATGATTCTCGAT TTTGTTGTAATAAATCCACCACGCTGCACGGAAGATTTTGA GATGAATCAAAATATACCTGATTTCATATTAATGCGAGTCGT
 1125 1170 1215

 GTACCACTCCGAATTGTTATGGACTATGCAATGTATGGAGAAAAG AGAGGTAAGGTGATTTCTGATAAAGTTGCTACATGATGATAAAAAGT AAAACAAAGGTAAATCCATCGAAAAATTTGTTGACGGCTATAAACTA
 1260 1305 1350

 GATGAACAAATATTGGTGATTATCCACACGCGTATACGTTTCG CCCTTTATTGCTGCTGTTACCCACTAACAGCAAAAAATCAAGAAATGG GTAAATAGTGGTTGGGATTGGATGAAGAATAAGAAAGAGAGTTAT
 1395 1440 1485

 TTCAGTGTAGTTATAATCTTTTAACTATGTTTATTTTAAACAGG AATTGGTGAACCGGATTTCCAGATGAAAAAAGATACAAAGTCCA ATAAATTTAGAAGTACAATCTGAATTTGAAGAACAGGATTAAGAT
 1530 1575 1620

 TGTATGAATACCAAAATTTAAATTTGATATTTTAAATATAG ATAAATAATTAGCAGCAACTTATATAGTAAAAAACCTAGGAATTT ATAATTTCTACGTTTTTACTTAAAGATAAACTATATGGATTAG
 1665 1710 1755

 ATAAACCATTTGGGGTGGAAATAAATAATGTACCGATTGGAAGT TGATGTAATAATAGCTT
 1800

Fig. 2. Nucleotide sequence of the gene for Endo-K from *Bacillus* sp. KSM-330 and the deduced amino acid sequence of the encoded protein. The nucleotide sequence of the non-transcribed strand is given from 5' to 3', and the deduced amino acid sequence is shown under the nucleotide sequence. Putative -35 and -10 regions of the promoters are indicated by broken underlining. The putative ribosome-binding site is underlined with double broken underlining. Horizontal arrows indicate terminator-like, inverted-repeat sequences. The deduced amino acid sequence identical to the amino-terminal sequence of the Endo-K protein (Ozaki & Ito, 1991) is indicated by a line under the amino acid sequence.

of the Endo-K protein secreted by *Bacillus* sp. KSM-330 (Ozaki & Ito, 1991), and the sequence from amino acids 53 to 55 (Val-Ser-Ala) resembles the recognition site of signal peptidases (Perlman & Halvorson, 1983). The Endo-K protein in *Bacillus* sp. KSM-330 may possibly be cleaved by a signal peptidase at the bond between Ala 55 and Val 56 during secretion across the cytoplasmic membrane. However, the site of cleavage is far from the carboxy-terminus of the hydrophobic region and the possible signal peptide of 55 amino acid residues seems somewhat longer than other reported signal peptides of endoglucanases from *Bacillus* (Fukumori *et al.*, 1986a; MacKay *et al.*, 1986; Robson & Chambliss, 1987). The amino-terminus of Endo-K could, therefore, be processed after secretion by a proteolytic enzyme. The molecular mass of the mature enzyme was calculated to be 46090 Da, based upon the deduced amino acid sequence from amino acids 56 to 463. This size is slightly larger than the 42 kDa determined by SDS-PAGE of Endo-K that was purified from *Bacillus* sp. KSM-330 (Ozaki & Ito, 1991), suggesting that the carboxy-terminal region of Endo-K may be subjected to proteolysis.

The endoglucanase activity of the purified Endo-K from *Bacillus* sp. KSM-330 is completely abolished by the presence of *N*-bromosuccinimide at low concentrations, and at least one Trp residue seems to be involved in the mechanism of action of this enzyme (Ozaki & Ito, 1991). The titrated number of total Trp residues in the enzyme molecule, 11.4, is very close to the number of Trp residues, 12, deduced from the nucleotide sequence of the gene.

Homology of the amino acid sequence with those of other enzymes

The deduced amino acid sequence of the Endo-K protein was compared with those of enzymes, reported to date, from various strains of *Bacillus* (Fukumori *et al.*, 1986a, b; MacKay *et al.*, 1986; Nakamura *et al.*, 1987; Robson & Chambliss, 1987; Fukumori *et al.*, 1989; Baird *et al.*, 1990; Ozaki *et al.*, 1990), *Clostridium* (Béguin *et al.*, 1985; Grépinet & Béguin, 1986; Joliff *et al.*, 1986; Hall *et al.*, 1988; Schwarz *et al.*, 1988; Zappe *et al.*, 1988; Faure *et al.*, 1989; Jauris *et al.*, 1990), *Cellulomonas* (Nakamura *et al.*, 1986; Wong *et al.*, 1986), *Ruminococcus* (Ohmiya *et al.*, 1989; Poole *et al.*, 1990; Wang & Thomson, 1990), *Butyrivibrio* (Berger *et al.*, 1989; Hazlewood *et al.*, 1990), *Pseudomonas* (Hall & Gilbert, 1988), *Erwinia* (Guiseppe *et al.*, 1988), *Streptomyces* (Nakai *et al.*, 1988) and *Trichoderma* (Penttilä *et al.*, 1986; Saloheimo *et al.*, 1988). The sequence from amino acids 56 to 443 of Endo-K exhibited 30% homology to that of the *celA* endoglucanase from *Clostridium thermocellum* NCIB10682 (Béguin *et al.*, 1985). In addition, 25% homology in terms of

Fig. 3. Homology between the amino acid sequences of endoglucanases from *Bacillus* sp. KSM-330, *C. thermocellum* NCIB10682 (*celA*) and *C. uda* CB4.

amino acid sequence was observed between Endo-K (from amino acids 130 to 273) and the endoglucanase from *Cellulomonas uda* CB4 (Nakamura *et al.*, 1986). No such homology was observed between Endo-K and other endoglucanases from *Bacillus*. This negative result suggests that the gene for Endo-K has evolved from a different ancestral gene than those for other reported enzymes from *Bacillus*. Henrissat *et al.* (1989) compared the amino acid sequences of cellulolytic enzymes from various origins by means of hydrophobic cluster analysis (Gaboriaud *et al.*, 1987) and classified them into six families (family A through family F). According to their criteria, Endo-K seems to belong to family D, because the endoglucanases from *C. thermocellum* NCIB 10682 and *Cellulomonas uda* CB4 have been classified as belonging to this family. A number of genes for endoglucanases from *Bacillus* have been sequenced, and some of the proteins (Fukumori *et al.*, 1986a, b; MacKay *et al.*, 1986; Nakamura *et al.*, 1987; Robson & Chambliss, 1987) have been classified as members of family A, and others (Fukumori *et al.*, 1989; Baird *et al.*, 1990; Ozaki *et al.*, 1990) appear to belong to the same family because they are similar to the enzymes in family A. Therefore, Endo-K is the first case of an enzyme from *Bacillus* that belongs to family D.

The amino acid sequences from amino acids 130 to 156 (region I) and from amino acids 189 to 207 (region II) of Endo-K appear to be well conserved when suitably aligned with sequences of endoglucanases in family D, as shown in Fig. 3. This result implies that some of the conserved amino acids may play important roles in the action of these endoglucanases. Among the conserved amino acids, the Trp residue found in region II was notable, in that at least one Trp residue has been proved to be involved in the mechanism of the action of Endo-K (Ozaki & Ito, 1991). The *celA* endoglucanase has a carboxy-terminal domain that consists of a direct repeat of 23 amino acids (Béguin *et al.*, 1985), as is conserved in other endoglucanases from *C. thermocellum* (Grépinet & Béguin, 1986; Joliff *et al.*, 1986; Hall *et al.*, 1988). These domains are unnecessary for both the catalytic activity

(Hall *et al.*, 1988) and binding to cellulose (Durrant *et al.*, 1991), and are separated from a catalytic domain by sequences which are rich in Pro, Thr and Ser residues. Such sequences of the reiterated amino acids and the Pro-Thr-Ser are absent in the Endo-K molecule, suggesting that this enzyme may be a single domain protein.

The aim of this study was to explain the difference in pH optima between the acid and alkaline endoglucanases of *Bacillus*. We expected that the acid endoglucanase, Endo-K, of *Bacillus* sp. KSM-330 would show some homology to the alkaline endoglucanase of *Bacillus* sp. KSM-635 (Ozaki *et al.*, 1990) and to other alkaline and neutral endoglucanases of *Bacillus* reported to date (Fukumori *et al.*, 1986a, b; MacKay *et al.*, 1986; Nakamura *et al.*, 1987; Robson & Chambliss, 1987; Fukumori *et al.*, 1989; Baird *et al.*, 1990). Interestingly, Endo-K exhibits no homology to these alkaline or neutral enzymes. It is clear that at least one Trp residue is involved in an action of this acid endoglucanase and of the alkaline endoglucanase from *Bacillus* sp. KSM-635 (Ozaki *et al.*, 1990; Yoshimatsu *et al.*, 1990), regardless of the difference in pH optima. We are now attempting to identify the Trp residue(s) that is necessary for the action of acid and alkaline endoglucanases by site-directed mutagenesis.

References

- ADHYA, S. & GOTTESMAN, M. (1978). Control of transcription termination. *Annual Review of Biochemistry* **47**, 967–996.
- AU, K.-S. & CHAN, K.-Y. (1987). Purification and properties of the endo-1,4- β -glucanase from *Bacillus subtilis*. *Journal of General Microbiology* **133**, 2155–2162.
- BAIRD, S. D., JOHNSON, D. A. & SELIGY, V. L. (1990). Molecular cloning, expression, and characterization of endo- β -1,4-glucanase genes from *Bacillus polymyxa* and *Bacillus circulans*. *Journal of Bacteriology* **172**, 1576–1586.
- BÉGUIN, P., CORNET, P. & AUBERT, J.-P. (1985). Sequence of a cellulase gene of the thermophilic bacterium *Clostridium thermocellum*. *Journal of Bacteriology* **162**, 102–105.
- BERGER, E., JONES, W. A., JONES, D. T. & WOODS, D. R. (1989). Cloning and sequencing of an endoglucanase (*endI*) gene from *Butyrivibrio fibrisolvens* H17c. *Molecular and General Genetics* **219**, 193–198.
- BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513–1523.
- DHILLON, N., CHHIBBER, S., SAXENA, M., PAJANI, S. & VADEHRA, D. V. (1985). A constitutive endoglucanase (CMCase) from *Bacillus licheniformis*-1. *Biotechnology Letters* **7**, 695–697.
- DOI, R. H. & WANG, L.-F. (1986). Multiple procaryotic ribonucleic acid polymerase sigma factors. *Microbiological Reviews* **50**, 227–243.
- DURRANT, A. J., HALL, J., HAZLEWOOD, G. P. & GILBERT, H. J. (1991). The non-catalytic C-terminal region of endoglucanase E from *Clostridium thermocellum* contains a cellulose-binding domain. *Biochemical Journal* **273**, 289–293.
- FAURE, E., BELAICH, A., BAGNARA, C., GAUDIN, C. & BELAICH, J.-P. (1989). Sequence analysis of the *Clostridium cellulolyticum* endoglucanase-A-encoding gene, *celCCA*. *Gene* **84**, 39–46.
- FUKUMORI, F., KUDO, T., NARAHASHI, Y. & HORIKOSHI, K. (1986a). Molecular cloning and nucleotide sequence of the alkaline cellulase gene from the alkalophilic *Bacillus* sp. strain 1139. *Journal of General Microbiology* **132**, 2329–2335.
- FUKUMORI, F., SASIHARA, N., KUDO, T. & HORIKOSHI, K. (1986b). Nucleotide sequences of two cellulase genes from alkalophilic *Bacillus* sp. strain N-4 and their strong homology. *Journal of Bacteriology* **168**, 479–485.
- FUKUMORI, F., KUDO, T., SASIHARA, N., NAGATA, Y., ITO, K. & HORIKOSHI, K. (1989). The third cellulase of alkalophilic *Bacillus* sp. strain N-4: evolutionary relationships within the *cel* gene family. *Gene* **76**, 289–298.
- GABORIAUD, C., BISSERY, V., BENCHETIT, T. & MORNON, J. P. (1987). Hydrophobic cluster analysis: an efficient new way to compare and analyse amino acid sequences. *FEBS Letters* **224**, 149–155.
- GRÉPINET, O. & BÉGUIN, P. (1986). Sequence of the cellulase gene of *Clostridium thermocellum* coding for endoglucanase B. *Nucleic Acids Research* **14**, 1791–1799.
- GUISEPPI, A., CAMI, B., AYMERIC, J.-L., BALL, G. & CREUZET, N. (1988). Homology between endoglucanase Z of *Erwinia chrysanthemi* and endoglucanases of *Bacillus subtilis* and alkalophilic *Bacillus*. *Molecular Microbiology* **2**, 159–164.
- HAGER, P. W. & RABINOWITZ, J. C. (1985). Translational specificity in *Bacillus subtilis*. In *The Molecular Biology of the Bacilli*, vol. II, pp. 1–32. Edited by D. A. Dubnau. Orlando: Academic Press.
- HALL, J. & GILBERT, H. J. (1988). The nucleotide sequence of a carboxymethyl-cellulase gene from *Pseudomonas fluorescens* subsp. *cellulosa*. *Molecular and General Genetics* **213**, 112–117.
- HALL, J., HAZLEWOOD, G. P., BARKER, P. J. & GILBERT, H. J. (1988). Conserved reiterated domains in *Clostridium thermocellum* endoglucanases are not essential for catalytic activity. *Gene* **69**, 29–38.
- HAZLEWOOD, G. P., DAVIDSON, K., LAURIE, J. I., ROMANIEC, M. P. M. & GILBERT, H. J. (1990). Cloning and sequencing of the *celA* gene encoding endoglucanase A of *Butyrivibrio fibrisolvens* strain A46. *Journal of General Microbiology* **136**, 2089–2097.
- HENIKOFF, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**, 351–359.
- HENRISSAT, B., CLAEYSSENS, M., TOMME, P., LEMESLE, L. & MORNON, J.-P. (1989). Cellulase families revealed by hydrophobic cluster analysis. *Gene* **81**, 83–95.
- JAURIS, S., RÜCKNAGEL, K. P., SCHWARZ, W. H., KRATZSCH, P., BRONNENMEIER, K. & STAUDENBAUER, W. L. (1990). Sequence analysis of the *Clostridium stercorarium celZ* gene encoding a thermoactive cellulase (Avicelase I): identification of catalytic and cellulose-binding domains. *Molecular and General Genetics* **223**, 258–267.
- JOHNSON, W. C., MORAN, C. P., JR. & LOSICK, R. (1983). Two RNA polymerase sigma factors from *Bacillus subtilis* discriminate between overlapping promoters for a developmentally regulated gene. *Nature, London* **302**, 800–804.
- JOLIFF, G., BÉGUIN, P. & AUBERT, J.-P. (1986). Nucleotide sequence of the cellulase gene *celD* encoding endoglucanase D of *Clostridium thermocellum*. *Nucleic Acids Research* **14**, 8605–8613.
- MACKAY, R. M., LO, A., WILICK, G., ZUKER, M., BAIRD, S., DOVE, M., MORANELLI, F. & SELIGY, V. (1986). Structure of a *Bacillus subtilis* endo- β -1,4-glucanase gene. *Nucleic Acids Research* **14**, 9159–9170.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MESSING, J. (1983). New M13 vectors for cloning. *Methods in Enzymology* **101**, 20–78.
- MÉZES, P. S. F. & LAMPEN, J. O. (1985). Secretion of proteins by bacilli. In *The Molecular Biology of the Bacilli*, vol. II, pp. 151–183. Edited by D. A. Dubnau. Orlando: Academic Press.
- MILLER, G. L., BLUM, R., GLENNON, W. E. & BURTON, A. L. (1960). Measurement of carboxymethylcellulase activity. *Analytical Biochemistry* **2**, 127–132.
- MORAN, C. P., JR., LANG, N., LEGRICE, S. F. J., LEE, G., STEPHENS, M., SONENSHAIN, A. L., PERO, J. & LOSICK, R. (1982). Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. *Molecular and General Genetics* **186**, 339–346.

- NAKAI, R., HORINOCHI, S. & BEPPU, T. (1988). Cloning and nucleotide sequence of a cellulase gene, *casA*, from an alkalophilic *Streptomyces* strain. *Gene* **65**, 229–238.
- NAKAMURA, K., MISAWA, N. & KITAMURA, K. (1986). Sequence of a cellulase gene of *Cellulomonas uda* CB4. *Journal of Biotechnology* **4**, 247–254.
- NAKAMURA, A., UOZUMI, T. & BEPPU, T. (1987). Nucleotide sequence of a cellulase gene of *Bacillus subtilis*. *European Journal of Biochemistry* **164**, 317–320.
- OHMIYA, K., KAJINO, T., KATO, A. & SHIMIZU, S. (1989). Structure of a *Ruminococcus albus* endo-1,4- β -glucanase gene. *Journal of Bacteriology* **171**, 6771–6775.
- OKOSHI, H., OZAKI, K., SHIKATA, S., OSHINO, K., KAWAI, S. & ITO, S. (1990). Purification and characterization of multiple carboxymethyl cellulases from *Bacillus* sp. KSM-522. *Agricultural and Biological Chemistry* **54**, 83–89.
- OZAKI, K. & ITO, S. (1991). Purification and properties of an acid endo-1,4- β -glucanase from *Bacillus* sp. KSM-330. *Journal of General Microbiology* **137**, 41–48.
- OZAKI, K., SHIKATA, S., KAWAI, S., ITO, S. & OKAMOTO, K. (1990). Molecular cloning and nucleotide sequence of a gene for alkaline cellulase from *Bacillus* sp. KSM-635. *Journal of General Microbiology* **136**, 1327–1334.
- PENTTILÄ, M., LEHTOVAARA, P., NEVALAINEN, H., BHIKHABHAI, R. & KNOWLES, J. (1986). Homology between cellulase genes of *Trichoderma reesei*: complete nucleotide sequence of the endoglucanase I gene. *Gene* **45**, 253–263.
- PERLMAN, D. & HALVORSON, H. O. (1983). A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. *Journal of Molecular Biology* **167**, 391–409.
- POOLE, D. M., HAZLEWOOD, G. P., LAURIE, J. I., BARKER, P. J. & GILBERT, H. J. (1990). Nucleotide sequence of the *Ruminococcus albus* SY3 endoglucanase genes *celA* and *celB*. *Molecular and General Genetics* **223**, 217–223.
- ROBSON, L. M. & CHAMBLISS, G. H. (1987). Endo- β -1,4-glucanase gene of *Bacillus subtilis* DLG. *Journal of Bacteriology* **169**, 2017–2025.
- SAITO, H. & MIURA, K. (1963). Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochimica et Biophysica Acta* **72**, 619–629.
- SALOHEIMO, M., LEHTOVAARA, P., PENTTILÄ, M., TEERI, T. T., STÄHLBERG, J., JOHANSSON, G., PETTERSSON, G., CLAEYSSENS, M., TOMME, P. & KNOWLES, J. K. C. (1988). EGIII, a new endoglucanase from *Trichoderma reesei*: the characterization of both gene and enzyme. *Gene* **63**, 11–21.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.
- SCHWARZ, W. H., SCHIMMING, S., RÜCKNAGEL, K. P., BURGSCWAIER, S., KREIL, G. & STAUDENBAUER, W. L. (1988). Nucleotide sequence of the *celC* gene encoding endoglucanase C of *Clostridium thermocellum*. *Gene* **63**, 23–30.
- SHIKATA, S., SAEKI, K., OKOSHI, H., YOSHIMATSU, T., OZAKI, K., KAWAI, S. & ITO, S. (1990). Alkaline cellulases for laundry detergents: production by alkalophilic strains of *Bacillus* and some properties of the crude enzymes. *Agricultural and Biological Chemistry* **54**, 91–96.
- SMITH, L. M., SANDERS, J. Z., KAISER, R. J., HUGHES, P., DODD, C., CONNELL, C. R., HEINER, C., KENT, S. B. H. & HOOD, L. E. (1986). Fluorescence detection in automated DNA sequence analysis. *Nature, London* **321**, 674–679.
- TEATHER, R. M. & WOOD, P. J. (1982). Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Applied and Environmental Microbiology* **43**, 777–780.
- TEWARI, H. K. & CHAHAL, D. S. (1977). Growth and cellulase formation by *Bacillus* sp. *Indian Journal of Microbiology* **17**, 23–26.
- TINOCO, I., JR, BORER, P. N., DENGLER, B., LEVINE, M. D., UHLENBECK, O. C., CROTHERS, D. M. & GRALLA, J. (1973). Improved estimation of secondary structure in ribonucleic acids. *Nature New Biology* **246**, 40–41.
- WANG, W. & THOMSON, J. A. (1990). Nucleotide sequence of the *celA* gene encoding a cellodextrinase of *Ruminococcus flavefaciens* FD-1. *Molecular and General Genetics* **222**, 265–269.
- WONG, W. K. R., GERHARD, B., GUO, Z. M., KILBURN, D. G., ANTHONY, R., WARREN, J. & MILLER, R. C., JR (1986). Characterization and structure of an endoglucanase gene *cenA* of *Cellulomonas fimi*. *Gene* **44**, 315–324.
- YOSHIMATSU, T., OZAKI, K., SHIKATA, S., OHTA, Y., KOIKE, S., KAWAI, S. & ITO, S. (1990). Purification and characterization of alkaline endo-1,4- β -glucanases from alkalophilic *Bacillus* sp. KSM-635. *Journal of General Microbiology* **136**, 1973–1979.
- ZAPPE, H., JONES, W. A., JONES, D. T. & WOODS, D. R. (1988). Structure of an endo- β -1,4-glucanase gene from *Clostridium acetobutylicum* P262 showing homology with endoglucanase genes from *Bacillus* spp. *Applied and Environmental Microbiology* **54**, 1289–1292.