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Cloning and sequencing of an endo-β-1,4-glucanase gene *mcen*A from *Micromonospora cellulolyticum* 86W-16

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SUMMARY

Endo-β-1,4-glucanase gene *mcen*A of *Micromonospora cellulolyticum* 86W-16 was cloned, and the nucleotide sequence was determined. An open reading frame (ORF) of 1374 bases, coding for a peptide (McenA) of 457 amino acids and 46742 Da, was found. It is preceded by a Gram-positive type of ribosome-binding site and followed by an imperfect inverted repeat. A putative signal peptide containing 23 amino acids is at the N-terminus and a linker region possessing 37 amino acids is in the midpart of McenA. The N-half of McenA functions as the catalytic domain and the C-half might serve as a cellulose-binding domain (CBD). Deletion of the latter did not decrease the CMCase activity of McenA. Significant similarity (70%) was found between the amino acid sequences of McenA and MbcelA, an endoglucanase from *Microbispora bispora*.

INTRODUCTION

Actinomycetes are dominant in the biodegradation of cellulose and hemicellulose in heated cellulosic wastes. Investigation on the mechanisms of enzymatic degradation of cellulose was initiated more than 30 years ago, and molecular cloning and sequencing of individual genes encoding cellulases have proved to be a useful approach for this goal. Application of actinomycetes necessitates an understanding of the action and regulation of their cellulases. Therefore, a number of cellulase genes have been cloned from *Microbispora bispora* [26], *Thermomonospora fusca* [11], *Thermomonospora curvata* [20] and an alkalophilic strain of *Streptomyces* [17]; much progress has been achieved [25].

Most members of the genus *Micromonospora* possess cellulolytic enzymes [14]. The genus is a promising microbial source for cellulose recycling. We describe in this paper the molecular cloning and nucleotide sequence of an endoglucanase gene, designated *mcen*A, from a new isolate, *Micromonospora cellulolyticum* 86W-16. Significant similarity has been found between amino acid sequences deduced from nucleotide sequences of *mcen*A and *mbcel*A, an endoglucanase-encoding gene from *Microbispora bispora* [4].

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MATERIALS AND METHODS

Bacterial strains, DNA vectors and enzymes

M. cellulolyticum 86W-16 was isolated from lake mud in Fuzhou, PR China. *Escherichia coli* HB101 and TG1 were used as hosts for different purposes, HB101 for plasmid pAT153, and TG1 for both plasmid pUC19 and phage DNAs M13BM20/21 that served as vectors for DNA sequencing. M13BM20/21 was purchased from Boehringer Mannheim (Germany). Enzymes from various sources were used according to descriptions provided by manufacturers.

Isolation and analysis of DNAs

Chromosomal DNA of *M. cellulolyticum* 86W-16 was obtained following the procedure 1 of Hopwood et al. [10]. GYPS (1% glucose, 0.5% yeast extract, 0.5% polypeptone, 0.15% soybean meal, 0.05% KH_2PO_4, 0.1% $K_2HPO_4 \cdot 3H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$, pH 7.2) was used as cultivation medium for *M. cellulolyticum* 86W-16 from spores to vegetative mycelia. A 70-h shaking period at 37 °C was followed by 2.5 h additional shaking after adding sterile sucrose and glycine to final concentrations of 25% (w/v) and 0.5% (w/v), respectively. Plasmids in *E. coli* were isolated using the boiling method of Maniatis et al. [15]. Isolation of fragmented DNAs from low-melting-temperature agarose was performed by using a DNA Purification Matrix Kit (Bio Rad, Richmond, CA, USA) or the usual phenol/chloroform technique. DNAs were analyzed by horizontal submarine agarose/ethidium bromide electrophoresis with λDNA/*Pst*I fragments as DNA molecular weight standards.

Construction of a genomic library

*Bam*HI-generated 2–7 kb fragments from *M. cellulolyticum* 86W-16 chromosomal DNA were recovered from low-melting-temperature agarose, ligated with T4 DNA ligase to *Bam*HI prelinearized and CIP dephosphorylated pAT153, and transformed into *E. coli* HB101 competent cells prepared by the calcium chloride method [2]. Ampicillin-resistant (Ap^r) and tetracycline-sensitive (Tc^s) transformants were harvested.

Screening for carboxymethylcellulase-positive clones

Carboxymethylcellulase-positive clones were screened by hydrolytic-zone formation on M9 [15] agar medium supplemented with 0.3% CMC·Na and 100 μg ml^{-1} Ap overlaid on 1.5% aqueous agar prehardened in Petri dishes. Before adding 0.1% (w/v) Congo Red solution to visualize the hydrolytic-zone formation [24], clones were incubated at 37 °C for 12–48 h.

Southern hybridization

Probe DNA (10 ng) was labelled with α-^{32}P-CTP (1–3 μCi) by using a Random Primer Labelling Kit (Fermentas, Lithuanian Republic). A VacuGene™ XL Vacuum Blotting System (Pharmacia-LKB, Sweden) was applied to blot DNAs from electrophoresed agarose gels to nylon membranes (Hybond™-N membrane, Amersham, UK). Prehybridization and hybridization experiments were carried out in accordance with procedures presented by Maniatis et al. [15]. After supplementing prehybridization and hybridization solutions with 25% (v/v) of formamide, a moderate temperature (37 °C) was used for prehybridization (2–12 h) and hybridization (12–48 h). Membranes after hybridization were washed at 42 °C with occasional shaking. Autoradiograms were prepared by incubating membranes with X-ray films (Kodak) at −70 °C for 12–48 h.

Gene sequencing

Fragments to be sequenced were recovered from low-melting-temperature agarose gels and subcloned into M13 vectors BM20 and BM21 precleaved with proper restrictases. Resulting subclones with inserts of interest were harvested from white plaques on transformation plates [2] containing isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). Deletion as a convenient technique was also applied to prepare some templates. Single-stranded template DNAs were sequenced by the dideoxy chain termination method [22] using the cycle sequencing technique on the Techne PHC-2 Thermal Cycler (Techne (Cambridge) Ltd, Duxford, Cambridge, UK) with fluorescent primers for the Applied Biosystem Model 370A DNA Sequencing System. The kit, for use with this procedure, includes Taq polymerase and c^7dGTP.

Computer analysis

Sequences were compiled and ordered using computer programs described by Staden [23]. Programs DNA-SUN and DNASIS (Pharmacia-LKB) were used for all analyses of DNA sequences in the text. Homologies with other amino acid sequences in the GeneBank DataBase were detected by dot plot analysis.

RESULTS

A partial *Bam*HI digest of *M. cellulolyticum* 86W-16 genomic DNA in the size range of 2–7 kb was ligated into *Bam*HI-cut vector pAT153. This ligation was transformed into *E. coli* HB101 competent cells and plated onto LB agar with selection pressure of Ap (100 μg ml^{-1}). A total of 4000 Ap^r clones were obtained, and 1000 clones presenting Tc^s were further screened for CMCase production by the Congo Red method. Only one clone, designated Clone 72, showed a visible hydrolytic zone (Fig. 1). Plasmid pCCT72 was isolated from Clone 72 and digested with *Bam*HI. A 2.4-kb foreign DNA insert was found in addition to pAT153 DNA. We considered that the 2.4-kb insert contained one active gene, termed *mcen*A, coding for a complete endoglucanase, or a catalytic domain of an endoglucanase. In order to make sure that *mcen*A was located in the cloned 2.4-kb fragment, pCCT72 was deleted from Clone 72 with acridine orange at a subinhibitory concentration of 150 μg ml^{-1} in LB. Three selected clones were Ap^sTc^s, contained no plasmid, showed microscopic rod-shaped cells and formed blue colonies on MacConkey agar; no CMCase activity was detected. However pCCT72 retransformation of these cured clones reestablished CMCase activity, supporting the above suggestion.

A *M. cellulolyticum* 86W-16 genomic origin of the 2.4-kb insert was confirmed by a Southern hybridization experiment between the 2.4-kb insert DNA and a *Bam*HI digest of *M. cellulolyticum* 86W-16 chromosomal DNA. Both purified 2.4-kb DNA and λDNA/*Pst*I digests were labelled with α-^{32}P-CTP respectively. DNAs in an electrophoresed agarose/ethidium bromide gel (Fig. 2(I)) were blotted onto a piece of nylon membrane. A membrane strip corresponding to the lane containing the λDNA/*Pst*I digest was cut out from the blotted membrane, and hybridized to the λDNA/*Pst*I probe; meanwhile, the remainder of the membrane was hybridized to the 2.4-kb DNA probe. The two pieces of membrane were recombined just before autoradiography started. The autoradiogram (Fig. 2(II)) showed a unique positive band of 2.4 kb from the *Bam*HI-digest of the *M. cellulolyticum* 86W-16 genome, consistent with the R_F value of the 2.4-kb insert DNA band as positive

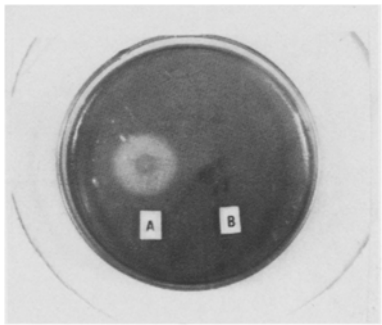

Fig. 1. CMCase activity in halo visualized with Congo Red in Clone 72. A, Clone 72; B, *E. coli* HB101 harboring pAT153.

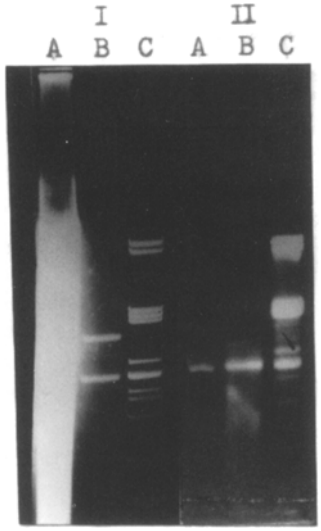

Fig. 2. Southern hybridization between the *mcen*A gene (as probe) and the *Bam*HI digest of chromosomal DNA of *M. cellulolyticum* 86W-16. I, Ethidium bromide-stained 0.75% agarose gel; II, Autoradiogram. Lane A, *Bam*HI digest of chromosomal DNA of *M. cellulolyticum* 86W-16; Lane B, *Bam*HI digest of pCCT72 DNA and Lane C, λDNA/*Pst*I digest as DNA molecular weight standards.

control. This result shows that the 2.4-kb insert harboring the *mcen*A gene originated from the *M. cellulolyticum* 86W-16 genome. The uniqueness of the positive band suggests that *mcen*A shows little homology to other CMCase genes in *M. cellulolyticum* 86W-16.

The 2.4-kb insert was mapped with more than 30 restrictases varying in restriction sites, and a sequencing strategy was subsequently set up (Fig. 3). When this insert was subcloned into the *Bam*HI site of pUC19, two constructions, opposite in orientation under the control of the *lac* promoter, expressed *mcen*A gene activity at very different levels (unpublished data). This was helpful for determination of the open reading frame (ORF) when sequencing was initiated from the *Sac*I site and carried out in two directions.

Fragments for dideoxy sequencing were separated and subcloned into proper sites of the M13 vectors BM20 and BM21. Nucleotide sequences of all adjacent fragments should sufficiently overlap and two strands must be complementary. The sequencing results are shown in Fig. 4. Computer analysis of the nucleotide sequence for the potential protein coding region identified only one long ORF extending from position 189 to 1562. We have assumed that potential initiation codons could be ATG or GTG. Only one ATG codon was observed, at position 921. This is the reason that we could not obtain results with ^{35}S-methionine using the maxi-cell technique. Alternative potential codons including GTG at positions 168, 189, 231, 264 and 294 on the 5′ end of the ORF. A potential start codon for translation at position 189 was preceded nine nucleotides upstream by the sequence 5′-AGGAGG-3′ (positions 174–179) and is likely to represent a ribosome-binding site. We have concluded that the GTG at position 189 is the correct start site for the *mcen*A structural gene. Hence, the structural part of the *mcen*A gene consists of 1374 bp, which codes for a protein with a molecular mass of 46742 Da. Computer analysis identified a potential transcription terminator. Only one base downstream of the translation stop codon TGA was observed, a sequence which is predicted to form an imperfect stem-loop structure having a stem length of 15 bp and a 2-bp loop similar to rho-independent terminators from *E. coli* [21]. No promoter-like sequence was included between the *Sma*I site and the start codon even though a *Sma*I–*Nru*I fragment constructed in the *Sma*I site of pUC19 could express CMCase activity in *E. coli* without promotion by P*lac* (unpublished data). A possible explanation [5] is that there was readthrough transcription from the vector. The ORF frequently used G (30.06%) and C (41.19%). Mol % (G + C) of *mcen*A (71.25) fell into the range of 70–75 (Tm) reported for *Micromonospora* chromosomal DNA [12].

Alignment of the amino acid sequence of McenA deduced from the *mcen*A gene sequence is shown in Fig. 4. The N-terminus of McenA contains a putative signal sequence of 23 amino acids sharing many of the features of protein

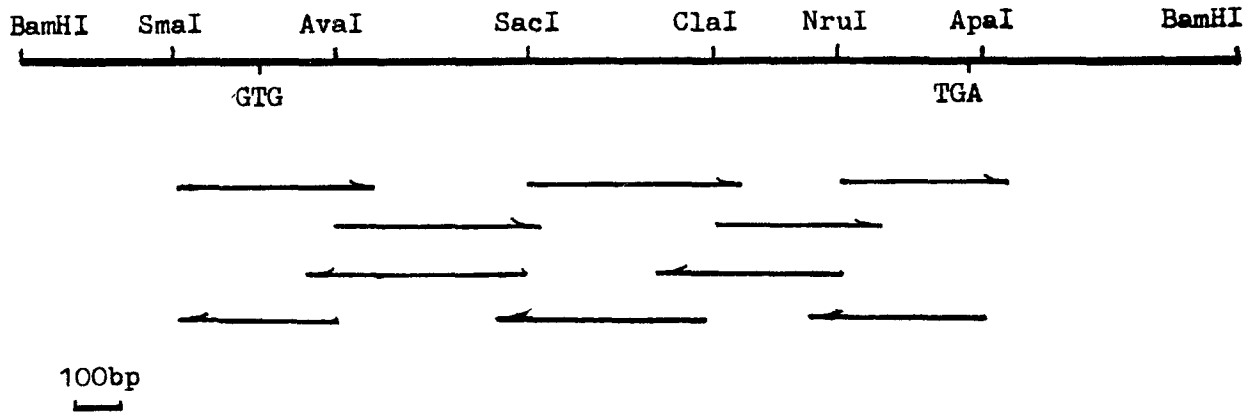

Fig. 3. Restriction map and sequencing strategy for *mcen*A gene.

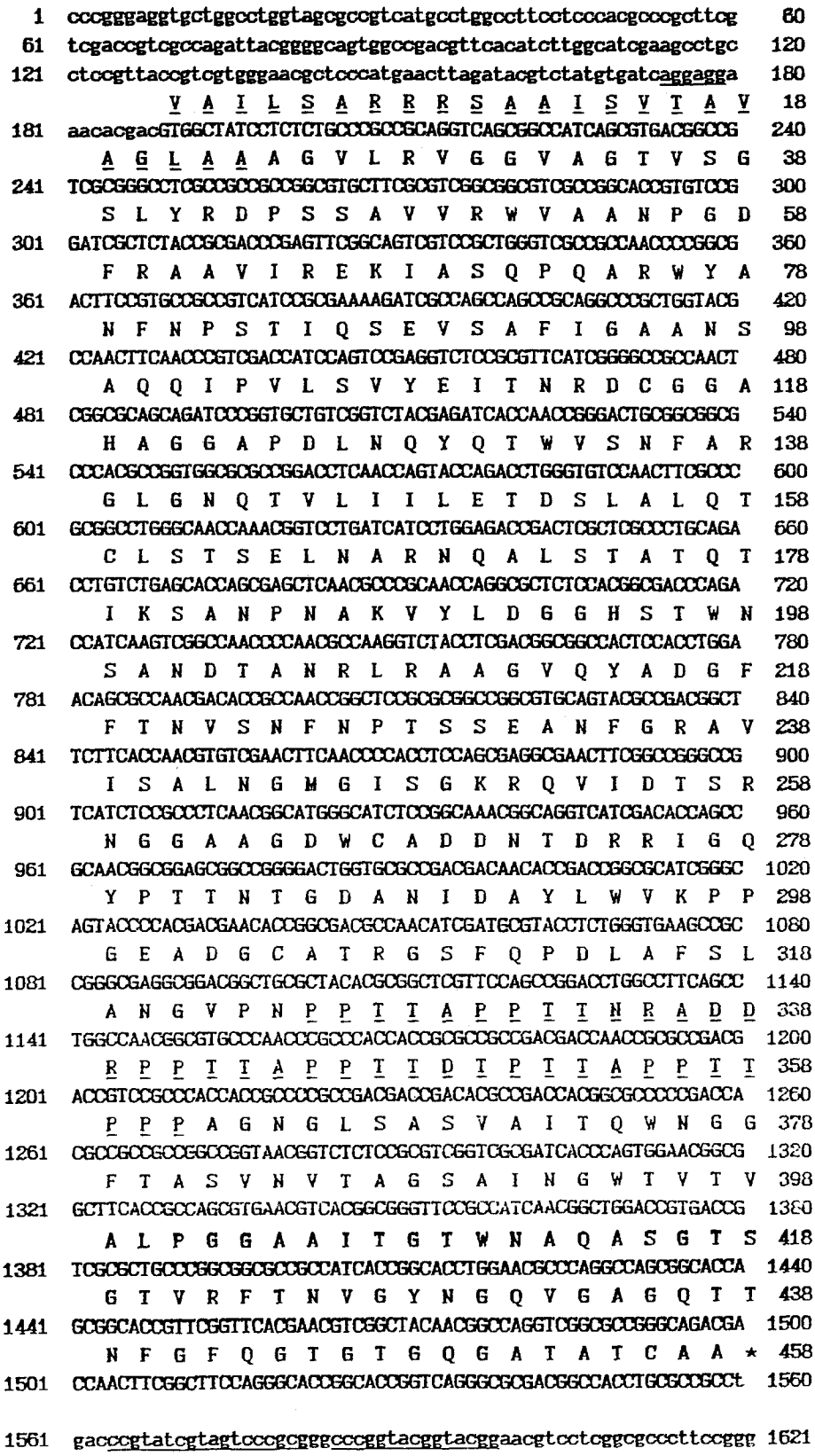

Fig. 4. Nucleotide sequence of *mcen*A and the deduced amino acid sequence of McenA. The putative SD sequence AGGAGG and the palindromic sequence downstream of the 3′ end of the ORF are underlined. The putative signal and linker regions are underlined with a dashed line.

signal sequences: 1–3 positively-charged amino acids at the N-terminus, followed by a hydrophobic region of about 10–15 amino acids [18], and an ala-ala-↓-ala processing site [13]. A linker comprising 37 amino acids, rich in proline and serine/threonine (Fig. 4), divides McenA into N- and C-halves (a catalytic domain and a cellulose-binding domain). Removal of 87 (90%) amino acids from the C-terminus of McenA did not lower the CMCase halo-forming capacity (unpublished data). It thus appears that the catalytic and cellulose-binding domains are located on N- and C-halves of McenA respectively.

Significant similarity was found between the complete amino acid alignments of McenA and MbcelA, an endoglucanase from *Microbispora bispora* [4]. It showed that 70% of McenA amino acids and 71% of MbcelA amino acids were similar (45% identical) (Fig. 5). A Dot Matrix Homology Plot result shows the high degree of similarity (Fig. 6).

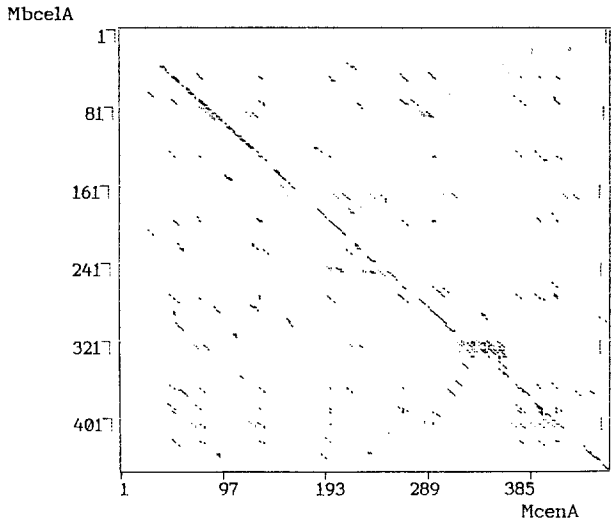

Fig. 6. Dot Matrix Homology Plot indicating the homology between MbcelA and McenA. Segment length = 6; Min score = 65; Scale = 2; MbcelA 1–456; McenA 1–457.

DISCUSSION

An endo-β-1,4-glucanase gene has been cloned from *M. cellulolyticum* 86W-16. The gene, *mcen*A was sequenced and its product McenA was deduced. An ORF of 1374 bases is flanked by a sequence AGGAGG nine bases upstream of the translation start codon and an imperfect inverted repeat only one base downstream of the stop codon. The high G + C content (71.25%) of *mcen*A fits the typical data (70–75%) for *Micromonospora* chromosomal DNA.

McenA (457 amino acids (aa), 46742 Da) possesses a putative signal peptide (23 aa) at its N-terminus, a linker (37 aa) rich in proline and serine/threoine, a catalytic domain and a cellulose-binding domain. The result of the C-terminus (87 aa) deletion predicts that the N-half (301 aa) and the C-half (96 aa), connected by a linker, function in cellulose catalysis and binding respectively.

A variety of conserved sequences have been found among cellulases from the same and from closely related bacteria [6,25] and among other Gram-negative and Gram-positive genera [6]. Interestingly, all the similarities were lower than 60%. Although some similarities of over 60% were reported between/among certain domains of cellulases from different sources [3,7,8,9,16,19] and between nucleotide sequences of two genes, *E*2 of *Thermomonospora fusca* and *mbcel*A [25], when integrated amino acid alignments of these cellulases were compared, similarities were lower than 60%.

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MbcelA   1   MSRIRRFLATALAAATAGVGAIVTAIASAGPAHAYDSPFYVDPQSNAAKWVAANPNDPRTPVIRDRIAAVPTGRWFANYNPSTVRAEVDAYVGAAAAAGK
             :* ** * : * **:* :  : * *      * ** *  :****** * *  ***: **  * : **: **: ****:  ** *: *** *
McenA    1   VAILSARRRSAAISVTAV-AGLAA-AGVLRVGGVAGTVSGSLYRDPSSAVVRWVAANPGDFRAAVIREKIASQPQARWYANFNPSTIQSEVSAFIGAANSAQQ

MbcelA 101   IPIMVVYAMPNRDCGGPSAGGAPNHTAYRAWIDEIAAGLRNRPAVIILEPDALPIMTNCMSPSE-QAEVQASMAYAGKKFKAASSQAKVYFDAGHDAWVP
             **: ** : ****** *****  * *:  * ** *  :**** * * : * *:* ** * ** :  *  * *  **** *: **  *
McenA  102   IPVLSVYEITNRDCGGAHAGGAPDLNQYQTWVSNFARGLGNQTVLIILETDSLALQT-CLSTSELNARNQA-LSTATQTIKSANPNAKVYLDGGHSTWMS

MbcelA 200   ADEMASRLRGADIANSADGIALNVSNYRYTSGLISYAKSVLSAI-G-A-SHLRAVIDTSRNGNGPLGSEWC--DPPGRATGTWSTTIDTGDPAIDAFLWIK
             * : * ***: * :   ***  ****:  **   ::: *:**  * : *  * ******** *  * :**  *  *  *  ** *** ***:**: *
McenA  200   ANDTANRLRAAGV-QYADGFFTNVSNFNPTSSEANFGRAVISALNGMGISGKRQVIDTSRNG-GAAG-DWCADDNTDRRIGQYPTTNTGDANIDAYLWVK

MbcelA 295   PPGEADGCIATPGVFVPDRAYELAMNAA-PPTYSPSPTPS----TPSPSPSQSDPGSPSPSPSQPPAGRACEATYALVNQWPGGFQAEVTVKNTGSSPIN
             ******** ** * * ** *: **     ***  * *       *:  * : * :  * : **** :  *: *:  ** *** * * *  *: * **
McenA  297   PPGEADGC-ATRGSFQPDLAFSLANGVPNPPTTAPPTTNRADDRPPTTAPPTTIDIPTTAPPTTPPPAGNGLSASVAI-TQWNGGFTASVNV--TAGSAIN

MbcelA 390   GWTVQWTLPSGQSITQLWNGDLSTSGSNVTVRNVSWNGNVPAGGSTSFGFLGSGTGQLSSSITCSA
             ****  ** * **  **:  * :  *  **  ** * ** : * *** *: ****  : ** *
McenA  393   GWTVTVALPGGAAITGTWNAQASGTSGTVRFTNVGYNGQVGAGQTTNFGFQGTGTGQ-GATATCAA
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Fig. 5. Amino acid sequence similarity between endoglucanases MbcelA and McenA. The putative signals and linkers of MbcelA and McenA are underlined. Identical and similar amino acid residues are respectively indicated with '*' and ':'.

With regard to cellulases, an unprecedented amino acid similarity (70%) and identity (45%) were found when McenA was compared to MbcelA. In addition, the extreme correspondence in arrangement of protein functional regions such as signals, linkers, catalytic domains and CBDs (Fig. 5) indicates a good commonality between McenA and MbcelA. In the light of the high similarity and identity reported in this report, in spite of the fact that *Micromonospora* and *Microbispora* are two different genera in *Micromonosporaceae*, our data should be helpful when the evolution of these two genera is considered. Interestingly, no homology of DNA was noticed between *mcen*A and other cellulase-coding genes in the cellulase complex of *M. cellulolyticum* 86W-16 (Fig. 2), as well as those of *M. cellulolyticum* 86W-6 (unpublished data), a strain close to *M. cellulolyticum* 86W-16 in morphology, physiology, biochemistry and taxonomy [1]. Thus, our previous strategy for cloning other cellulase genes from *M. cellulolyticum* 86W-16 with the *mcen*A probe should be modified, and shot-gun methods should be used instead.

With the worldwide aim of recycling cellulosic wastes by the use of cellulases or microbial cellulase producers, much effort has been made to understand the catalytic mechanisms by molecular cloning of individual cellulase genes. *Micromonospora*, as a gene donor, excretes a complex of endo-, exoglucanases and β-glucosidase. Leading to the goal, our task will deal with understanding those results and catalytic mechanisms of the enzyme complex by cloning other cellulase-encoding genes, and, if possible, application of *mcen*A through ideal expression systems including hyper-expression vectors and applicable microbial hosts.

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