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Purification and Characterization of the CelB Endoglucanase from *Streptomyces lividans* 66 and DNA Sequence of the Encoding Gene

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The endoglucanase CelB isolated from culture filtrates of *Streptomyces lividans* IAF9 has an M_r of 36,000. With carboxymethyl cellulose as the substrate, the V_{max} and K_m values are 110 IU/mg of enzyme and 1.3 mg/ml, respectively. Comparison of primary amino acid sequences classifies CelB in the H family of cellulases.

Streptomyces lividans secretes all the hydrolytic enzymes required to degrade the lignocellulosic biomass (6). To date, we have reported the cloning and the sequences of genes coding for three xylanases (5, 11, 15, 18), one cellulase (17), and one mannanase (1). This was achieved in the homologous system of *S. lividans* by functional complementation of a cellulase- and xylanase-negative pleiotropic mutant (11). In this paper, a second β-1,4-endoglucanase (CelB) is described as part of the study of the expression of the genes involved in biomass degradation by *S. lividans*.

Production and isolation of endoglucanase. Optimal expression of cellulase by the multicopy recombinant plasmid pIAF9 was achieved by its introduction into the endocellulase-negative mutant *S. lividans* IAF8.83 (14). The preparation of microbial cultures, the culture conditions, and the culture media have been described previously (17). The mycelium was removed by centrifugation of the fermentation broth, and the supernatant (5 liters) was passed through a DEAE column (12 by 5 cm). The cellulase was eluted with 0.2 M NaCl–20 mM piperazine buffer, pH 6.0. The active fractions were combined, and $(NH_4)_2SO_4$ was added to a final concentration of 1 M. This solution was passed through a phenyl-Sepharose CL-4B column (10 by 5 cm). The cellulase was eluted with a reverse gradient of $(NH_4)_2SO_4$ (1 to 0 M). The active fractions, eluted in 40% ethylene glycol, were purified by high-performance liquid chromatography as described previously (17). Five liters of fermentation broth yielded about 1.3 mg of pure enzyme. *S. lividans* IAF9 produces very low levels of CelB (0.1 IU/ml) in comparison with the levels of enzyme produced by the CelA-producing clone IAF74 (12 IU/ml) (17). Considering that the activity of CelB is about 10-fold lower than that of CelA, the total production of CelB is still 10-fold less. This may be attributed to a difference in the promoter strength, because the same vector and the same culture conditions were used for both clones.

Characterization of the endoglucanase. The apparent molecular mass of CelB was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7) and estimated to be 36 kDa (Fig. 1A). Analytical isoelectric focusing of CelB indicated a pI of 4.2 (data not shown). The cloned endoglucanase from *S. lividans* IAF9 was compared with those of *S. lividans* 1326 and 3131 (the wild-type strain containing pIJ702). SDS-PAGE followed by a Western blot (immunoblot) (11) probed with anti-CelB antibodies showed a single 36-kDa band in the wild type. As expected, the mutant strain *S. lividans* 8.83 did not react, and the purified CelA showed no immunological cross-reaction (Fig. 1B). The Michaelis-Menten constants, determined at pH 6.5 and 50°C with carboxymethyl cellulose (CMC) as the substrate, are a V_{max} of 110 IU/mg of enzyme and a K_m of 1.3 mg/ml. CelB has more affinity for CMC than CelA does, although its V_{max} is 10-fold lower than that of CelA. By comparison of the ratios of the pseudo-second-order constant ($V_{max} \cdot K_m^{-1}$) for the substrate, we observed that CelA has a fourfold-higher relative specificity for CMC. CelB showed no activity against synthetic substrates such as methylumbelliferyl-cellobiopyranoside or *p*-nitrophenyl-cellobiopyranoside or against xylan. CelB did not hydrolyze Avicel, but acid-swollen Avicel was degraded into cellobiose, cellotriose, and cellotetraose. However, CelA produces cellobiose from Avicel (17). Only qualitative differences were observed in the hydrolysis of cello-oligosaccharides by the two cellulases (data not shown).

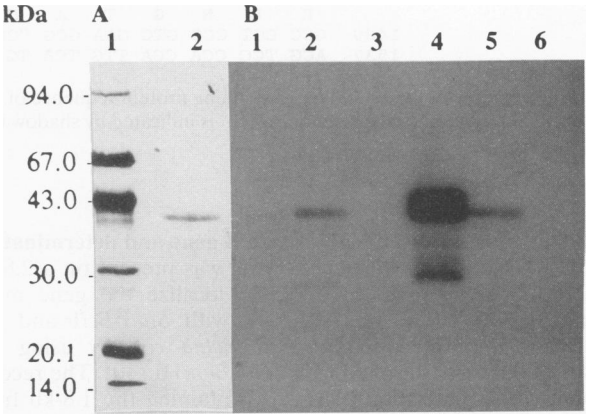

FIG. 1. (A) SDS-PAGE of purified CelB from *S. lividans* IAF9 stained with Coomassie brilliant blue. Lanes: 1, molecular mass standards; 2, purified CelB (5 μg). (B) Western blot analysis of culture filtrate proteins (100 μg) with antiendoglucanase antibodies. Lanes: 1, *S. lividans* 1326 (wild type); 2, *S. lividans* 3131 (wild-type strain containing pIJ702); 3, *S. lividans* IAF8.83 (cellulase-negative mutant); 4, *S. lividans* IAF9/8.83; 5, purified CelB (2 μg); 6, purified CelA (10 μg).

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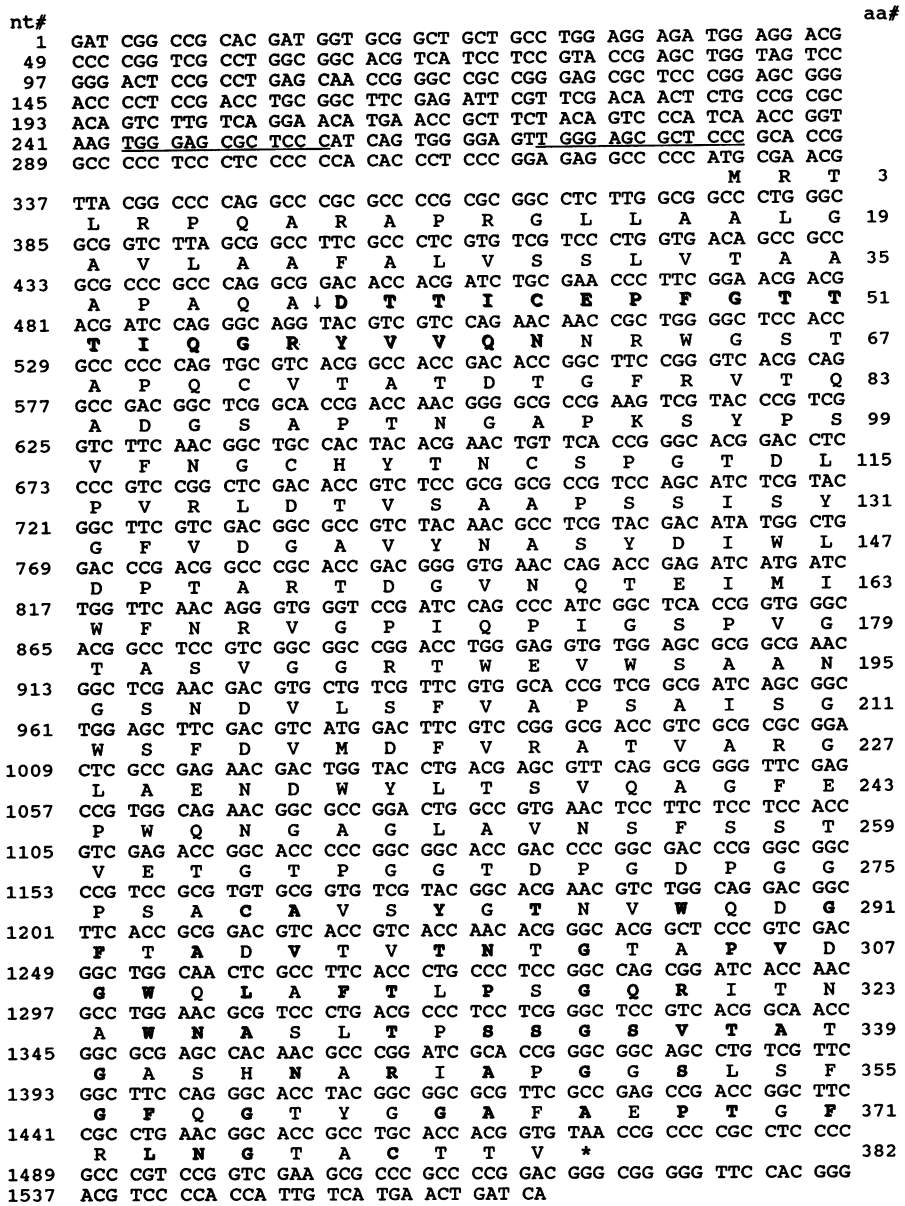

FIG. 2. DNA sequence and corresponding protein sequence of the *celB* gene of *S. lividans*. The two 13-bp inverted repeats are underlined. The N-terminal sequence of the mature protein is indicated by shadow type. The amino acids homologous with those of CelA in the CBD are indicated in boldface.

Subcloning of the endoglucanase B gene and determination of its DNA sequence. The *celB* gene was present on a 2.8-kb insert in pIAF9 (14). In order to localize the gene more precisely, the insert was digested with *Sph*I-*Bcl*I and the fragment was subcloned in *Escherichia coli* by using the phagemid pTZ19 digested with *Sph*I-*Bam*HI (10). The recombinant clone, harboring pIAF210 containing the 1.8-kb fragment, still produced a clearing zone on CMC plates when the plates were stained with Congo red after growth (17). The nucleotide sequence of *celB* was established by using plasmid pIAF210 with the Automated Laser Fluorescent (ALF) DNA Sequencer and ALF Fragment Manager software, version 2.5 (Pharmacia LKB). The N-terminal amino acid sequence of the purified mature extracellular CelB was determined (DTTI CEPFGTTTIQGRYVVQN) and is shown shaded in Fig. 2. It validated the *celB* nucleotide sequence. From nucleotide (nt) 328 to nt 1470, an open reading frame encodes a 39,265-Da polypeptide, 3,955 Da of which accounts for the signal peptide. Thus, the processed secreted CelB (35,310 Da) corresponds closely to the M_r of 36,000 estimated for the native enzyme by SDS-PAGE. Two 13-bp inverted repeats are present between nt 244 and 283 (underlined in Fig. 2). A single copy of such a sequence was found at the 5′ end of *celA* from *S. lividans* (17). Identical sequences have also been found immediately upstream of the coding regions of the cellulase gene of *Streptomyces* strain KSM-9 (12); the *celE2*, *celE4*, and *celE5* genes of *Thermomonospora fusca* (8); and *celA1* of *Streptomyces halstedii* JM8 (3). A gel shift assay showed that, upon cellulase

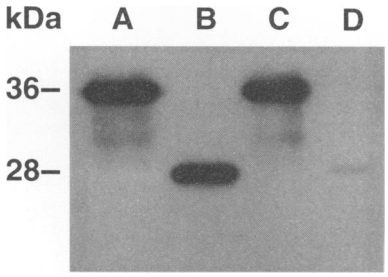

FIG. 3. Analysis of the CBD of CelB. Avicel was incubated separately with CelB (36 kDa) and a truncated version of CelB (28 kDa). The protein fraction bound to Avicel was analyzed by SDS-PAGE and then by Western blotting and immunodetection with anticellulase antibodies. Lanes: A, CelB control (10 μg); B, truncated CelB control (10 μg); C, CelB bound to Avicel; D, truncated CelB bound to Avicel.

induction, this DNA sequence is the target of a positive activator (9). Thus, in *S. lividans*, *celA* and *celB* genes, which are not linked in an operon, seem to be regulated by a similar activator-repressor-type mechanism. Surprisingly, two rarely used TTA codons specifying leucine were found in the CelB signal sequence. In streptomycetes, these rare codons are encountered in temporally regulated genes such as genes expressed during sporulation and antibiotic synthesis (2). The finding of these codons in *celB* may explain the poor expression of this gene by the wild-type strain. However, cellulase activity is immediately detected in the culture supernatant of *S. lividans* growing on cellulose as the substrate (6), and the presence of CelB was shown by immunodetection on a Western blot (data not shown). This indicates that the cellulase genes are involved in the primary metabolism.

Sequence homology analysis. Comparison of the predicted amino acid sequence of CelB with amino acid sequences of other cellulases was performed by using the TFASTA program of the Genetics Computer Group and the GenBank and EMBL data banks. The first 211 amino acids (aa) of the N terminus of endoglucanase B of *S. lividans* show 29% identity and more than 50% similarity with only one cellulase: the FI-CMCase of *Aspergillus aculeatus* (13). On the basis of the hydrophobic cluster analysis of the amino acid sequence by Gilkes et al. (4), the endoglucanase B of *S. lividans* belongs to family H. The cellulose-binding domain (CBD) which is located at the carboxy-terminal end of CelB resembles that of CelA of *S. lividans* (17). The highly conserved residues, mainly the cysteine (C), the asparagine (N), and the tryptophan (W), described for other CBDs (4) are shown in Fig. 2. The catalytic domain of CelB is separated from its CBD by a linker sequence (aa 212 to 225). During CelB (36 kDa) purification, a 28-kDa protein fraction exhibiting cellulase activity was also isolated. The N-terminal sequence was identical to that of CelB, indicating that this protein resulted from the carboxy-terminal end degradation of CelB. The difference in M_r, 8,000, represented the removal of approximately 70 amino acid residues of the 103 aa involved in the CBD of CelB, as deduced from the sequence. The results of cellulose-binding assays (16) with CelB and its truncated version are shown in Fig. 3. CelB bound strongly to cellulose, while a very small amount of the 28-kDa cellulase remained attached to the substrate. This experiment confirmed the presence of a functional CBD in CelB. We now plan to disrupt the cellulase genes in *S. lividans* in order to evaluate their respective functions during cellulose utilization.

Nucleotide sequence accession number. The *celB* sequence of *S. lividans* has been deposited in GenBank and carries the accession number U04629.

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