Lic16A of *Clostridium thermocellum*, a non-cellulosomal, highly complex endo-β-1,3-glucanase bound to the outer cell surface

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*Clostridium thermocellum* produces one major β-1,3-glucanase. Genomic DNA fragments containing the gene were cloned from two strains, DSM1237T (6848 bp) and F7 (9766 bp). Overlapping sequences were 99-9 % identical. The nucleotide sequences contained reading frames for a putative transposase, endo-β-1,3-1,4-glucanase CelC, a putative transcription regulator of the LacI type, β-1,3-glucanase Lic16A and a putative membrane protein. The licA genes of both strains encoded an identical protein of 1324 aa with a calculated molecular mass of 148 kDa. Lic16A is an unusually complex protein consisting of a leader peptide, a threefold repeat of an S-layer homologous module (SLH), an unknown module, a catalytic module of glycosyl hydrolase family 16 and a fourfold repeat of a carbohydrate-binding module of family CBM4a. The recombinant Lic16A protein was characterized as an endo-1,3(4)-β-glucanase with a specific activity of 2680 and 340 U mg⁻¹ and a Km of 0.94 and 2.1 mg ml⁻¹ towards barley β-glucan and laminarin, respectively. It was specific for β-glucans containing β-1,3-linkages with an optimum temperature of 70°C at pH 6.0. The N-terminal SLH modules were cleaved from the protein as well in *Escherichia coli* as in *C. thermocellum*, but nevertheless bound tightly to the rest of the protein. Lic16A was located on the cell surface from which it could be purified after fractionated solubilization. Its inducible production allowed *C. thermocellum* to grow on β-1,3- or β-1,3,1,4-glucan.

INTRODUCTION

Non-cellulosic carbohydrates constitute the second largest fraction of biomass on earth. In plants they are for example important structural components in the cell wall or nutritional components of the endosperm. However, they are extraordinarily heterogeneous, consisting of a number of different sugar residues which are partially derivatized and linked. Complex systems of distinct enzymes are necessary to hydrolyse each of their constituents. One fraction of these polysaccharides, β-1,3-glucan, can be isolated in pure form, for example from the reproductive structures of plants (callose), from the marine macro-alga *Laminaria saccharina* (laminarin), from the cell walls of yeasts and fungi (zymosan or schizophyllan) or as an exopolysaccharide from bacteria like *Alcaligenes faecalis* var. *myxogenes* (curdlan) (Harada, 1992). β-1,3-Glucosidic linkages are also found in the linear mixed-linkage β-glucans of the *Gramineae* (e.g. barley) or in the lichen *Cetraria islandica* (lichenan), alternating with β-1,4-linkages (30 : 70 %).

Bacteria hydrolyse β-1,3-linkages by specific endoglucanases. Some of them have been characterized, e.g. from *Bacillus* *circularis*, *Clostridium thermocellum*, *Rhodothermus marinus* or *Thermotoga maritima* (Schwarz et al., 1988a; Spilliaert et al., 1994; Zverlov et al., 1997a). They are biochemically classified either as 1,3-β-D-glucan glucanohydrolases (EC 3.2.1.39) or as 1,3-β-D-glucan 3(4)-glucanohydrolases (EC 3.2.1.6). Their sequences are homologous and belong to glycosyl hydrolase family GHF16 (Henriassat & Bairoc, 1996). But despite a similar protein fold, the β-1,3-glucanases cleave only β-1,3-linkages, whereas the β-1,3,1,4-glucanases hydrolyse the β-1,4-linkage adjacent to a β-1,3-linkage (Keitel et al., 1993).
The three-dimensional structure of some members of GHF16 has been resolved (Hahn et al., 1995; Krah et al., 1998). The β-1,3-glucanases produce a characteristic pattern of β-1,3-oligosaccharides, which are further degraded by β-glucosidases. In contrast to the highly specific β-glucanases, β-glucosidases degrade laminari-oligosaccharides (β-1,3) as well as cello-oligosaccharides (β-1,4) (Gräbnitz, et al. 1989; Zverlov et al., 1997b).

β-1,3-Glucanases have a commercial potential, for example for yeast extract production or the conversion of algal biomass to fermentable sugars. They also have, in combination with chitinases, antitumour activity for disease protection of plants (Nogi & Horikoshi, 1990). Partial hydrolysis of β-1,3,glucans can lead to biologically active products, for example to therapeutic biological response modifiers with antiviral, antitumour or related activity.

Hydrolysis of xylan takes place (Nochur et al., 1990; Schwarz, et al., 1985; Tuka et al., 1981). The DNA of the Lic16A(Cat) module with pLICA7 (5'-aagaaaatctgctgacgaagatgcaagg-3') and the DNA of the Lic16A(Cat) module with pLICA7 (5'-gctgtgacgaagatgcaagg-3') and pLICA8 (5'-aactactctattgtgtgtgatgg-3'). The resulting DNA fragments were cloned with the expression vector pQE32 (Qiagen). To amplify a 420 bp fragment of Orf1 with the PCR DIG Labeling Mix (Roche Diagnostics) the oligonucleotide primers pTra1 (5'-aagaaatctgctgacgaagatgcaagg-3') and pTra2 (5'-aactactctattgtgtgtgatgg-3') were used.

DNA sequences were determined from supercoiled double-stranded plasmid DNA for both strands by using the Thermosequenase Cycle Sequencing Kit (Amersham Biosciences) for extension of 5'-biotinylated oligonucleotide primers. DNA fragments were detected with the GATC 1500 Direct-Blotting-Electrophoresis apparatus using streptavidin-conjugated alkaline phosphatase and NBT-BCIP (nitro blue tetrazolium-5-bromo-4-chloro-3-indolylphosphatase) as chromogenic substrate (Roche Diagnostics). Sequence data were analysed, edited and compared with the DNAiNISORSI for Windows package (Hitachi Software Engineering). Nucleotide and protein sequence databases were screened using FASTA and BLAST software at the NCBI server (http://www.ncbi.nlm.nih.gov).

**METHODS**

**Strains and growth conditions.** E. coli XL-1 Blue was used for cloning and expression of clones. C. thermocellum DSM1237 (corresponds to ATCC 27405) was obtained from the German Collection of Micro-organisms (DSMZ) and strain F7 from the All-Russian Collection of Micro-organisms (VKMB 2203). Recombinant E. coli was grown at 37°C in Luria–Bertani broth (LB) containing ampicillin (100 μg ml⁻¹). Schizosaccharomyces pombe (DSM2791) was grown in LB. Bacillus subtilis MW10, which is deleted for the β-glucanase gene, was obtained from R. Bouriss and grown in LB (Wolf et al., 1995). dLB medium was LB containing 20 g glucose l⁻¹. C. thermocellum was grown in rubber-stoppered glass bottles with pre-reduced, anaerobic GS-2 medium containing 1% (w/v) cellulose or other carbohydrates at 60°C (Johnson et al., 1981).

**Recombinant DNA techniques.** Clones αLic7 and pCU309, containing the laminarinase gene of strains DSM1237 and F7, respectively, have been described previously (Schwarz, et al. 1985; Tuka et al., 1990). DNA preparation, cloning, restriction-endonuclease digestion, hybridization and PCR were carried out by standard procedures (Sambrook et al., 1989). The Expand-High-Fidelity PCR-System was used according to the recommendations of the manufacturer (Roche Diagnostics). The intact licA gene (pKL112) was amplified with primers pLICA1 (5'-aaccggatctataaacgggtagtaccagggagg-3') and pLICA4 (5'-cgccttatacactgactacggcagc-3'), and the DNA of the Lic16A(Cat) module with pLICA5 (5'-gtgctgtgacgaagatgcaagg-3') and pLICA8 (5'-aactactctattgtgtgtgatgg-3'). The resulting DNA fragments were cloned with the expression vector pQE32 (Qiagen). To amplify a 420 bp fragment of Orf1 with the PCR DIG Labeling Mix (Roche Diagnostics) the oligonucleotide primers pTra1 (5'-aacagaattctgcaagg-3') and pTra2 (5'-tactactatttactacttggttgg-3') were used.

Genomic libraries have previously been constructed from two C. thermocellum strains, DSM1237 and F7 (Schwarz et al., 1985; Tuka et al., 1990). Clones expressed in Escherichia coli were extensively screened for enzymic activities related to β-1,3-glucan (laminarin) and mixed-linkage glucan (lichenan) hydrolysis. In this paper we describe the primary structure of the gene licA, its product Lic16A and the flanking genomic area in both strains. We discuss a possible evolutionary mechanism of the extraordinarily complex β-glucanase Lic16A, the relation of the β-1,3- to the β-1,3,1,4-glucanases and the localization of the protein. We show growth of C. thermocellum on β-1,3-glucan and present a characterization of the purified recombinant and the native β-1,3-glucanase (laminarinase), which is thermostable and highly active.
ileX genes from *E. coli*, and the kanamycin resistance and p15A replication origin from pACYC177 (Chang & Cohen, 1978) for compatibility with the pBR322-derived expression plasmids in *E. coli*.

**Purification of the enzyme.** *E. coli* pKL112 (pQE32::licA)/pKL162 cells from an IPTG-induced 3 l culture in dLB with ampicillin (100 mg l\(^{-1}\)) and kanamycin (25 mg l\(^{-1}\)) were collected by centrifugation, washed twice in 20 mM Tris/HCl, pH 8-0, suspended in the presence of Benzonase and Pefa-Bloc protease inhibitor (both Merck) and disintegrated by two to six passages through a French pressure cell (AmlInCo) at 110 MPa with ice cooling. Completeness of disintegration was controlled microscopically. Cell fragments were removed by centrifugation (Sorvall SS34, 18,000 r.p.m., 30 min). The clarified cell extract was heated to 55°C for 30 min, centrifuged and filtered through a 0-2 μm membrane filter. The filtrate was applied to a Q-Sepharose FF anion-exchange column, equilibrated with the same buffer, and eluted with an increasing NaCl gradient (0-1000 mM). Protein fractions having β-glucanase activity were applied to a hydrophobic interaction chromatography column Phenyl-Sepharose HP 16/10 (Amersham Biosciences) in 1-2 M (NH\(_4\))\(_2\)SO\(_4\) and eluted with a decreasing gradient of (NH\(_4\))\(_2\)SO\(_4\) in 20 mM Tris/HCl, pH 8-0. The active fractions were applied in 100 mM NaCl, 20 mM Tris/HCl, pH 8-0 to a gel filtration on a Superdex 200 prep grade XK 16/60 column (Amersham Biosciences).

**Western blotting and protein sequencing.** SDS-PAGE slabs were blotted on PVDF membranes (Roche Diagnostics). Monoclonal anti-His-IgG (Qiagen) or polyclonal rabbit antibodies elicited against purified Lic16A(Cat) were used for detection. N-terminal sequences were determined from protein eluted from cut-out pieces of SDS gel electrophoresis slabs by Edman degradation using a Procise 492 protein sequencer (Applied Biosystems). The phenylthiohydantoin derivatives were identified by reversed-phase HPLC. Endoproteinase LysC (Roche Diagnostics) was used to digest degradation-resistant native Lic16A. The peptide mixture was separated by reversed-phase HPLC.

**Zymogram technique.** Proteins were separated by denaturing SDS-PAGE (10 % polyacrylamide) and renaturated by three successive washings in 50 mM phosphate buffer (pH 6-2) with and without i-propanol as described previously (Schwarz et al., 1987). The gel slabs were overlaid with agarose gel containing polymeric substrates (1 %, w/v), incubated at 55°C and stained with 0.05 % Congo Red (Sigma-Aldrich). The gel slab was overlaid with a hydrophobic interaction chromatography column Phenyl-Sepharose HP 16/10 (Amersham Biosciences) in 1-2 M (NH\(_4\))\(_2\)SO\(_4\) and eluted with a decreasing gradient of (NH\(_4\))\(_2\)SO\(_4\) in 20 mM Tris/HCl, pH 8-0. The active fractions were applied in 100 mM NaCl, 20 mM Tris/HCl, pH 8-0 to a gel filtration on a Superdex 200 prep grade XK 16/60 column (Amersham Biosciences).

**Enzymic assays and detection of hydrolysis products.** Enzyme aliquots in standard assays were incubated in succinate buffer (100 mM, pH 6-0) with 1 % (w/v) substrates at 60°C. Reducing sugars released from polymeric substrates were detected by the 3,5-dinitrosalicylic acid (DNSA) method (Wood & Bhat, 1988). Insoluble substrates were precipitated by centrifugation and the supernatant with DNSA. One unit of activity is defined as the release of 1 μmol glucose equivalent min\(^{-1}\). \(V_{\text{max}}\), \(K_m\) and \(K_a\) were determined from the slope of the linear portion of the reaction curve by measuring the initial velocity at varying concentrations of substrate at the optimum temperature and pH.

Mono- and oligosaccharides were separated by TLC on 0-2 mm silica gel 60 plates (Merck) in multiple runs in acetonitrile/water (80:20, v/v). Sugars were detected by spraying with a solution of 1 g diphenylamine and 1 ml aniline in 100 ml acetone plus freshly added 10 ml orthophosphoric acid. Colour was developed on heating at 120°C.

**Preparation of yeast cell walls.** For isolation of *Saccharomyces cerevisiae* cell walls, commercial bakers yeast was used. *Sch. pombe* cells were cultured and collected by centrifugation. Cells were washed by centrifugation, homogenized and disrupted by eight to twelve passages through a French Press (AmlInCo). The cell walls were sedimented by centrifugation, washed five times with pure water and lyophilized. No reducing sugars could be detected in the supernatant with DNA.

**Substrates.** Oat spelt xylan, carboxymethyl-cellulose, lichenan, CMC (low viscosity), laminarin, soluble starch and chitin were obtained from Sigma-Aldrich; barley β-glucan, pachyman and CM-pachyman from Megazyme; pustulan from Roth; curdlan from Wako; soluble starch from Merck; and Avicel CFI from Serva. Laminaridextrins as size standards for HPLC and TLC were obtained from Seikagaku. Phosphoric acid swollen cellulose was prepared from Avicel CFI by the method of Wood & Bhat (1988).

**RESULTS**

**The licA region in the genome of two C. thermocellum strains.**

Cosmid clone \(\lambda\) Lic7 expressing β-1,3-glucanase activity has been isolated previously from a genomic library of the *C. thermocellum* type strain DSM1237\(^T\) (Schwarz et al., 1985, 1988a). The nucleotide sequence of a subcloned DNA fragment was determined (5595 bp; accession no. X89732). It overlapped the previously obtained sequence of another subclone that encoded the endoglucanase gene celC (accession no. M19422; Schwarz et al., 1988b). The connected sequences cover 6698 bp (Fig. 1). To verify the unusual structure of the genes we also determined the corresponding sequence of clone pCU309 from strain F7, another strain of *C. thermocellum* (9760 bp; accession no. AJ307315). The F7 sequence covered that from DSM1237\(^T\) completely and extended beyond it at both ends (Fig. 1). The sequences were 99-9 % identical in the overlapping area, with the majority of the mismatches in intergenic regions.

**Fig. 1.** The CelC-Lic16A region in the genome of two *C. thermocellum* strains. (a) The location and transcription direction of the reading frames are indicated by arrows, the sequenced regions by black bars. The GenBank accession numbers are indicated: AJ307315 for *C. thermocellum* strain F7, M19422 and X89732 for DSM1237\(^T\). (b) Structure of Lic16A. LP, leader peptide.
The reading frames and the structure of the genes were equivalent and are described below for the longer F7 sequence.

Six ORFs were identified and all but Orf2 were preceded by potential Shine–Dalgarno sequences (Fig. 1). All frames were encoded on the same DNA strand. The corresponding protein sequences were almost completely conserved between the two strains, because most mismatches were in the third codon position and did not change the amino acid composition or the reading frames (as, for example, all mismatches in licA and celC). The celC gene region could be compared with a third sequence from strain F1, isolated in Japan (Sakka et al., 1991), which contained only four amino acid exchanges (of 343 aa residues) compared to the corresponding sequences of DSM1237\(^1\) and F7.

**Putative transposase gene**

The first reading frame in the F7 sequence (Orf1, bp 257–712) had a high sequence identity with IS605/IS200 transposases of family 17 (Pfam accession no. PF01797; Bateman et al., 2002), e.g. 92 % similarity to the C-terminal part of the Clostridium acetobutylicum transposase (aa 58–151; accession no. AE007849). However, the alignment of amino acid residues 29–151 with different transposases, including that of Clostridium perfringens or Thermotoga maritima, was perfect if a gap of 10 aa residues between L57 and K58 was introduced. It should be mentioned that the overall size of the homologous bacterial transposase genes was identical to the transposase gene proposed here.

To verify the presence of IS605/200-homologous DNA sequences in the genome of C. thermocellum F7, an amplified 420 bp DNA fragment corresponding to the well conserved C-terminal region was hybridized to three complete endonuclease restriction enzyme digests of genomic DNA from strain F7. The multitude of resulting bands of hybridizing DNA sequences indicated the presence of a complete endonuclease restriction enzyme digests of genomic DNA from strain F7. The multitude of resulting bands of hybridizing DNA sequences indicated the presence of a number of different insertion sites in the chromosome of strain F7 (Fig. 2). The intensity of the hybridizing bands may vary due to the occurrence of double and triple bands.

**Other reading frames**

Orf2 is dealt with in the Discussion section. Orf3 encodes the non-cellulosomal \(\beta\)-1,3-1,4-glucanase CelC (bp 2350–3381), which was described previously (Schwarz et al., 1988b). Orf4 shows up to 52 % similarity to bacterial transcription repressors of the GalR/LacI family over the whole length of the gene. Orf6 is truncated at the C terminus. It is up to 62 % similar to the N-terminal 322 aa residues of hypothetical membrane proteins, which consist of a leader peptide and eight putative transmembrane regions, e.g. from C. perfringens or Staphylococcus aureus.

**The modular structure of the licA gene**

Orf5 encodes laminarinase A and is called licA due to the high activity of the gene product on lichenan which it shares with LicB (Zverlov et al., 1994). A putative ribosome-binding site is located at base 4632 upstream of a coding sequence starting at ATG (base 4645) and leading to a large protein of 1323 aa (148 116 Da). licA is followed by a downstream 13 bp hairpin structure and a run of T residues, presumably a rho-factor independent transcription terminator. The translated amino acid sequence consists of a hydrophobic N terminus (25 aa) with the typical features of a signal peptide (von Heijne, 1986), three repeated S-layer homologous modules (SLH; aa 27–227), a module of no significant similarity to the databases which is connected via a short stretch of hydroxy-amino acids (PTS-box) to a catalytic module (aa 421–671), and followed by four consecutive carbohydrate-binding modules of family 4a (CBM4a) (Fig. 1b; Zverlov et al., 2001). The catalytic module aligns well with bacterial glycosyl hydrolase family 16 modules, which were found in \(\beta\)-1,3-glucanases and mixed-linkage glucanases of Bacillus, Thermotoga and other bacteria (Coutinho & Henrissat, 1999). Hence the gene product was named Lic16A and can be described by the structural formula LP/SLH/SLH/SLH/X/PT/GH16/CBM4a/CBM4a/CBM4a/CBM4a according to a recently proposed nomenclature (Henrissat et al., 1998).

**Heterologous expression and purification of Lic16A\(_{\text{DSM1237}}\)**

The codon usage in gene licA is as biased as it is in other genes of C. thermocellum, especially for the arginine codons...
AGG and AGA and the isoleucine codon AUA, for which tRNAs in *E. coli* are scarce. A succession of these codons in a heterologous gene impedes overexpression in *E. coli* host cells (Del Tito *et al.*, 1995; Zdanovsky & Zdanovskaia, 2000). Accordingly, the heterologous expression of *licA* in *E. coli* was low and IPTG induction resulted in severe growth retardation and heavy protein degradation. Expression of *licA* in *Bacillus subtilis* MW10 in the shuttle-vector pHPS9 resulted in similar difficulties (Haima *et al.*, 1990; Wolf *et al.*, 1995). In the presence of the helper plasmid pKL162, which supplemented the limiting IleX and ArgU tRNAs, the *licA*-mediated induction in *E. coli* was increased 10- to 12-fold and Lic16A protein was less degraded. Upon IPTG induction, the isolated catalytic module of Lic16A [LicA(Cat)] in plasmid pQE32 amounted to 50% of the soluble cell proteins (data not shown).

Lic16A<sub>DSM1237</sub> was purified from induced *E. coli* cells by liquid chromatography. In denaturing SDS-PAGE two major proteins forming bands of 120 and 29 kDa were detected which co-purified with the enzymic activity and could not be separated by gel filtration or affinity chromatography (data not shown, but similar to Fig. 3). The molecular mass calculated from calibrated gel filtration chromatography was about 130 kDa, identifying Lic16A as a monomeric enzyme. The 22 N-terminal amino acids of the 120 kDa protein band were determined by micro-sequencing (SIHFINTKIN RVVVNKTGVR IV) and verified that the 120 kDa protein was a fragment of Lic16A, truncated by its 266 N-terminal amino acids. Taking the processing site between aa residues 266 and 267, the calculated masses for the cleaved products would be 118-3 kDa for the C-terminal fragment (including the catalytic module) and 29-5 kDa for the N-terminal protein (including the leader peptide). This was corroborated by the experimental data (Fig. 3) and suggested a single, specific processing site, cleaving the complete 148 kDa protein, either containing the leader peptide or the His tag.

If the N terminus was split off the catalytic module, an N-terminal hexahistidyl tag added by cloning into vector pQE32 in the place of the leader peptide could help to purify the complete Lic16A protein of 148 kDa by metal-chelating affinity chromatography. But again the two bands of 120 and 29 kDa were obtained in equimolar amounts in denaturing SDS-PAGE as described above (Fig. 3). Only the 120 kDa band, but not the 29 kDa band, was active in zymograms with barley β-glucan and thus contained the catalytic module, whereas only the smaller polypeptide reacted with anti-His tag antibodies, identifying it as the N terminus of Lic16A. A band of the complete 148 kDa protein could not even be detected in the highly sensitive Western blot with anti-Lic16A(Cat)-antibodies. Despite the occurrence of two bands in denaturing SDS-PAGE we have purified a homogeneous protein: the protein obviously was quantitatively cleaved but the two fragments of Lic16A bound tightly to each other.

![Fig. 3. Recombinant Lic16A protein purified with His-tag affinity chromatography, denaturing SDS-PAGE and Western blotting. Lanes: 1, purified recombinant Lic16A stained with Coomassie blue (bands of 120 and 29 kDa); 2 and 3, Western blot with anti-Lic16A(Cat) (only 120 kDa; lane 2) and anti-His tag antibodies (only 29 kDa; lane 3). The size of marker proteins is indicated.](http://mic.sgmjournals.org)

**Biochemical characterization of Lic16A**

Purified recombinant Lic16A was capable of degrading barley β-1,3,1,4-glucan and with a lower reaction velocity, lichenan and laminarin (Table 1). Activity on pure β-1,3-glucans, e.g. pachyman, CM-pachyman and insoluble curdlan, was present but low. Activity towards *Sch. pombe* cell walls which also contain β-1,3-glucan, was obtained, but surprisingly not with *Sac. cerevisiae* cell walls. β-1,4-Glucans (like crystalline cellulose, acid swollen cellulose or CMC), other β-1,4-glycans (like xylan or chitin) or *p*-nitrophenyl-β-D-glycosides were not hydrolysed. This indicated that the enzyme catalysed the hydrolysis of β-1,3-glucosidic linkages and is a β-1,3-glucanase (laminarinase). A *V<sub>max</sub>* of 3175 and 417 U mg<sup>−1</sup>, a *k<sub>cat</sub>* of 7474 and 836 s<sup>−1</sup>, a *K<sub>m</sub>* of 0-94 and 2-11 mg ml<sup>−1</sup> and an apparent *k<sub>cat</sub> K<sub>m</sub><sup>−1</sup> of 7951 and 396 ml s<sup>−1</sup> mg<sup>−1</sup> were determined with the substrates barley β-glucan and laminarin, respectively, at 60˚C and pH 6-0.
**Table 1. Activity of purified recombinant Lic16A**

Assays were performed in 0.1 M phosphate-citrate buffer at pH 6.0 and 60°C. ND, Not detectable.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Relative activity (%)</th>
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<tbody>
<tr>
<td>Barley (β-1,3-1,4-glucan)</td>
<td>2680</td>
<td>100</td>
</tr>
<tr>
<td>Lichenin (β-1,3-1,4-glucan)</td>
<td>2407</td>
<td>90</td>
</tr>
<tr>
<td>Laminarin (β-1,3-1,6-glucan)</td>
<td>340</td>
<td>13</td>
</tr>
<tr>
<td>CM-pachymann (β-1,3-glucan)</td>
<td>86</td>
<td>3.2</td>
</tr>
<tr>
<td>Pachymann (β-1,3-glucan, insoluble)</td>
<td>0.43</td>
<td>0.02</td>
</tr>
<tr>
<td>Curdlan (β-1,3-glucan, insoluble)</td>
<td>29.4</td>
<td>1.1</td>
</tr>
<tr>
<td>Sch. pombe cell walls</td>
<td>1.14</td>
<td>0.04</td>
</tr>
<tr>
<td>Sac. cerevisiae cell walls</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

The optimum activity of Lic16A on barley β-glucan was observed at a temperature of 70°C and a pH of 6.0 with a second maximum at pH 9.0 (80 % of activity at pH 6.0) and 50 % of the activity between pH 5.5 and 10.0. Half of the Lic16A activity was left after 10 min pre-incubation without barley β-glucan at 70°C, but no significant inactivation was observed after 2 h at 65°C. Lic16A was severely inhibited by low concentrations (1–5 mM) of Hg, Fe, Zn and Cu ions, whereas no influence was detected with sulfhydrating (DTT) and complexing (EDTA) agents, or ethanol (<10 %), SDS (<20 mM) and NaCl (<50 mM).

The products of hydrolysis were primarily cello-tri- and tetraoside from barley β-glucan, cello-tri- and pentaoside from lichenan and laminari-bi-, tri- and tetraoside from laminarin. Limited amounts of glucose were obtained from laminarin only after prolonged incubation. The cellobio-oligosaccharides released from mixed-linkage glucans indicated that the β-1,3-linkages and not the β-1,4-linkages were cleaved. Time kinetics of laminarin degradation showed a gradual decrease of chain length of the resulting oligosaccharide products, as would be expected for an endo-mode of hydrolysis (data not shown).

**Production and localization of Lic16A in C. thermocellum**

*C. thermocellum* DSM1237T was inoculated on media with different polysaccharides as sole carbon source. Cells grew within 4 days to an optically dense culture on cellulbiose, Avicel, CMC, barley β-glucan and laminarin, but not (within 10 days) on xylan, insoluble curdlan, pustulan, yeast cell walls, chitin and starch. The cultures reached the highest density on laminarin and barley β-glucan: the protein yield of cell extracts was 50–100 % higher than on Avicel and even higher than on cellulbiose (Table 2). Unexpectedly, β-glucans containing β-1,3-linkages were the best substrates to sustain growth of *C. thermocellum*.

The specific activity of supernatants and cell extracts of all cultures was determined on β-1,3-glucan and barley β-1,3,1,4-glucan. Zymogram investigations had shown that mixed-linkage β-glucan is hydrolysed by all endoglucanases, whereas a comparison with Western blots revealed that Lic16A is the prevalent if not the only β-1,3-glucanase in *C. thermocellum* (see below). The ratio of the specific activities on laminarin and barley β-glucan can therefore be regarded as an indication for the specific expression of Lic16A: whereas this ratio was 0.6 and 11.3 % in the cell extracts of Avicel- and cellulbiose-grown cells, it was significantly higher in laminarin- and β-1,3,1,4-glucan-grown cultures (26.1 and 21.4 %), respectively, (Table 2). This indicates a specific induction of Lic16A expression by β-glucans containing β-1,3-linkages. This result was supported by Western blot experiments with antibodies prepared against purified Lic16A(Cat) where the amount of reacting protein increased upon growth on β-1,3,1,4-glucan and laminarin (Fig. 4). Fig. 4 also reveals that the larger band of native Lic16A had a molecular mass of 125 kDa, slightly higher than in recombinant *E. coli*. No 148 kDa protein was detected with Lic16A(Cat)-antibodies, neither in culture supernatants nor in cell extracts, indicating complete processing not only in *E. coli* but also within the *C. thermocellum* cell (prior to secretion), although probably at a different site. Bands with a smaller mass (92, 80 and 61 kDa) may be the result of further processing and roughly fit to a gradual release of the C-terminal CBM4a modules.

To investigate the localization of mature Lic16A and to purify the protein from the native *C. thermocellum* DSM1237T, cultures were raised on CMC which showed an eight times higher induction of β-1,3-glucanase activity than cellulbiose, but was much more affordable than

**Table 2. Enzymic activities in cell extracts of *C. thermocellum* cultures grown on different carbohydrate sources**

Cell-free extracts were prepared from aliquots of fully grown cultures (4 days) and protein content was estimated. Barley β-glucan and laminarin was digested with culture supernatants and reducing sugars were estimated with DNSA reagent (units as glucose equivalent).

<table>
<thead>
<tr>
<th>Carbohydrate source</th>
<th>Protein (mg ml⁻¹)</th>
<th>β-Glucan (U ml⁻¹)</th>
<th>Laminarin (U ml⁻¹)</th>
<th>Lam/Gluc (%)</th>
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<tbody>
<tr>
<td>Avicel (β-1,4)</td>
<td>3-63</td>
<td>122</td>
<td>0.75</td>
<td>0.6</td>
</tr>
<tr>
<td>Cellobiose (β-1,4)</td>
<td>5.15</td>
<td>13.3</td>
<td>1.5</td>
<td>11.3</td>
</tr>
<tr>
<td>CMC (β-1,4)</td>
<td>3.14</td>
<td>12.8</td>
<td>1.7</td>
<td>12.9</td>
</tr>
<tr>
<td>Barley β-glucan (β-1,3-1,4-glucan)</td>
<td>7.69</td>
<td>43.3</td>
<td>9.3</td>
<td>21.4</td>
</tr>
<tr>
<td>Laminarin [β-1,3-(1,6)-glucan]</td>
<td>5.97</td>
<td>29.8</td>
<td>7.8</td>
<td>26.1</td>
</tr>
</tbody>
</table>
β-1,3-glucan. During growth, cell-bound laminarinase activity increased proportionally with the optical density of the culture (OD600) and the cell protein content, and thus was not growth-phase-dependent (data not shown).

After 4 days of growth the cells were collected and washed differentially: 150 mM NaCl or distilled water released only 7% of the laminarinase activity, whereas 2 M guanidinium hydrochloride (GuHCl) solubilized Lic16A completely, but only 10% of the cell protein. No more laminarinase was washed off the cells by 5 M GuHCl and the cells did not retain β-1,3-glucanase activity. A control experiment showed that laminarinase activity recovered at least partially after 5 M GuHCl treatment and would have been detected if present. The 5 M GuHCl wash contained a dominant 130 kDa protein band, presumably the major S-layer protein (Lemaire et al., 1995). Western blots with anti-Lic16A(Cat)-antibodies and zymograms with barley β-glucan corroborated that Lic16A could be solubilized selectively from the cell surface by 2 M GuHCl treatment and contained an enzymically active protein of 125 kDa.

The comparison of Lic16A-Western blots and β-1,3-glucan zymograms revealed that Lic16A was the major if not the only β-1,3-glucanase present.

Purification of Lic16A from C. thermocellum and interaction with cellular proteins

The native Lic16A protein was purified from the 2 M GuHCl fraction by liquid chromatography as described for the recombinant Lic16A protein. A protein active on xylan was separated from Lic16A by anion-exchange chromatography (MonoQ; Amersham Biosciences). It was probably xylanase XynX, which is bound to the cell wall via its terminal SLH modules (Lemaire et al., 1995), similar to Lic16A. A hydrophobic interaction chromatography step finally resulted in a homogeneous native Lic16A protein. The amount of Lic16A represented 0·23% of the total cell-bound protein if purification losses were taken into account. Since the N terminus was blocked for Edman degradation, the identity of the purified protein with Lic16A was verified by sequencing the N terminus of an internal LysC-protease fragment: the amino acid residues KDGALVIEARKEDI were identified, corresponding to amino acid residues 468–481.

The SLH modules of extracellular proteins were previously shown to interact with cellular components (Lemaire et al., 1995). To investigate binding of Lic16A to specific proteins, C. thermocellum extracts were separated in denaturing SDS-PAGE, blotted on PVDF membranes and incubated in blocking buffer for 1 h with purified LicA protein. With the complete Lic16A, but not with Lic16A(Cat) which is devoid of SLH modules, three bands with a molecular mass of 110, 85 and 61 kDa were detected with anti-His IgG (Fig. 5). Faint bands which could be the result of unspecific binding were neglected. The major bands represent three proteins

![Fig. 4](http://mic.sgmjournals.org)

**Fig. 4.** Production of Lic16A by C. thermocellum DSM1237T upon growth on different polysaccharides. A 200 μl culture equivalent of clarified cell extract was subjected to denaturing SDS-PAGE; the gel was Western blotted with anti-Lic16A(Cat) antibodies. Lanes: 1, purified recombinant Lic16A (100 ng); 2–6, cell extracts from cells grown on cellobiose (lane 2), barley β-glucan (3), laminarin (4), CMC (5) and Avicel (6). The position of molecular mass marker proteins is indicated (pre-stained SDS-marker 7B; Sigma-Aldrich).

![Fig. 5](http://mic.sgmjournals.org)

**Fig. 5.** Interaction of cell proteins with the SLH module of Lic16A. Lanes: 1, cell extracts from C. thermocellum cells separated by SDS denaturing gel electrophoresis and incubated with 5 nmol His6-Lic16A; 2, cell extract, but incubated with His6-Lic16A(Cat) which is devoid of the SLH modules. Visualization was by detection with anti-His-tag IgG. Position of the marker proteins is indicated by arrows.
of the cell to which SLH modules of Lic16A are specifically binding.

**DISCUSSION**

The chromosomal region around licA and celC was sequenced in two different strains of *C. thermocellum*, the type strain DSM1237T (corresponds to ATCC 27405 and NCIB 10682), isolated in the USA, and strain F7, isolated in Armenia (former Soviet Union; Chuvilskaya et al., 1987). Despite the isolation from different continents, nucleotide sequences were surprisingly similar and the amino acid sequences of LicA and CelC were identical. This corroborates data obtained with other pairs of genes: LicB and ChiA in DSM1237T and F7 (Schimminig et al., 1992; Zverlov et al., 2002), two xylanase genes in strains ATCC 27405 and F1 (>99.3%), celllobiose phosphorylase Cbp in strains YM4 and F1 (99.6%) and CelK in strains F7 and YW20 (99.8%), which contain only a few differences. Thus the *C. thermocellum* strains isolated in the Northern hemisphere seem to be quite homogeneous, as was also suggested from the physiological characteristics of different isolates (McBee, 1954).

Two glycosyl hydrolase genes, celC and licA, are located on the same strand of the sequenced DNA fragments. Both genes encode proteins involved in non-cellulosomal β-glucan degradation. According to the arrangement of chromosomal DNA fragments and the restriction map obtained by DNA–DNA hybridization, plasmids pKS305 and pCU309 are located within a close distance (Guglielmi & Béguin, 1998). PKS305 contains the genes manB and celT (Kurokawa et al., 2002). It should be noted that according to a comparison of the restriction map given in Guglielmi & Béguin (1998) and the DNA sequences, the cellulosomal genes manB and celT are located downstream of orf6 (Fig. 1) and transcribed from the same DNA strand. A cluster of four genes involved in cellulose degradation is therefore located in this chromosomal area of *C. thermocellum*.

The two proteins CelC and LicA differ greatly in structure: Lic16A has a complex structure of one catalytic and eight non-catalytic modules, whereas CelC contains only a single (catalytic) module, like the majority of the extracellular hydrolases of the aerobic Gram-positive bacteria, e.g. the *Bacillus* amylases. The high complexity of the Lic16A structure is comparable to the most complex cellulosytic proteins from the non-cellulosomal cellulosome system of *Caldicellulosiruptor* sp. or its relative *Anaerocellum thermophilum* (Gibbs et al., 2000; Zverlov et al., 1998). A comparison between the two strains and partial resequencing of sequence differences between the genes made the correctness of the unusual structure of licA reliable beyond reasonable doubt.

It can be speculated that the arrangement of the completely different hydrolase structures in a narrow region of the *C. thermocellum* genome, CelC and LicA, is the result of the evolution of new catabolic genes by genetic recombination events, by which new catabolic pathways are assembled (Mahillon et al., 1999). Hence, it is not surprising that a transposase gene [at least a precursor or remnant of an insertion element (orf1)] was found to be located upstream of licA and celC in the genome of *C. thermocellum* F7. Similar arrangements of cellulosome gene clusters and a transposase gene can be observed in the chromosomes of *Clostridium cellulolyticum*, *Clostridium cellulovorans* and *C. acetobutylicum* (Lynd et al., 2002). At present there is no experimental evidence that the multiple copies of the IS605/200-type insertion elements are actively involved in the evolution of *C. thermocellum* or if orf1 is a IS200-transposase variant with an unusual N-terminal sequence. However, it should be mentioned that the DNA region immediately downstream of the putative transposase gene shows homology to the C-terminal part of the cell wall lysis gene cwUL of *Paenibacillus polymyxa* (orf2 in Fig. 1a), beginning with amino acid residue 68 (bp 954). The homology region begins with a typical hexameric sequence (5’-TTTAA-3’) which was reported to be the duplicated end of the IS605/200 element in *Helicobacter pylori* (Kersulyte et al., 1998). This suggests that an IS605/200 element (orf1) may have inserted in a cwUL-homologous gene in *C. thermocellum* and could have contributed to the module shuffling, resulting in the complex structure of nearby licA.

The licA gene was introduced into *E. coli*. Obviously, the initially low expression level was due to the occurrence of multiple codons for rare host aminoacyl-tRNAs clustered in the 5′ region of the gene upstream of the catalytic module. This problem was overcome by introducing a helper plasmid containing the underrepresented tRNA genes. A similar improvement upon introduction of the same helper plasmid was found in expressing the *C. thermocellum* genes licB or chiA, and the *Clostridium stercorarium* gene celY, and facilitated an efficient heterologous production with a high yield of unprocessed protein (unpublished observations).

In its biochemical characteristics Lic16A is a typical endo-β-1,3-glucanase of glycosyl hydrolase family 16 (EC 3.2.1.39) with an extraordinarily high activity on soluble mixed-linkage glucans and an affinity to barley β-glucan three times higher than to laminarin. The sequence of Lic16A has overall homology to the thermostable *R. marinus* β-1,3-glucanase BglA (42.8% identity in a 257 aa overlap) rather than to the *Bacillus*-type mixed-linkage β-glucanases, which cleave almost exclusively β-1,4-glycosyl linkages on 3-O-substituted gluco-pyranose units (Anderson & Stone, 1975; Henrisaat & Bairch, 1996; Spilliaert et al., 1994). The difference is reflected in its active site sequence which contains four additional amino acid residues (Fig. 6), as was found with the β-1,3-glucanases of *B. circulans* KCTC3004, *R. marinus* (LamR) or *Thermotoga maritima* (LamA) which have a similar substrate specificity and pattern of products (Yahata et al., 1990; Spilliaert et al., 1994; Aono et al., 1995; Lee & Chang, 1995; Zverlov et al., 1997a; Krah et al., 1998).
The SLH modules of bacterial exoenzymes, involved in intermolecular interaction and in binding to cell wall components, are usually located at the C terminus, whereas in Lic16A and in the alkaline cellulase of Bacillus KSM-635 they are found N-terminally (Ozaki et al., 1990). Lic16A indeed interacted with a number of cell proteins of a similar size as those described for OlpB in C. thermocellum or EngB in C. cellulovorans, which also contain SLH modules (Lemaire et al., 1995; Kosugi et al., 2002). However, it did not interact with the 130 kDa major S-layer component. The interacting proteins were not identified, but they could play a role in the surface binding of extracellular proteins. Besides binding to the cell envelope, the SLH modules interact with each other and seem to form a network of interacting polysaccharide-hydrolyzing enzymes on the cell surface, with Lic16A being a part of it (Lemaire et al., 1995).

The N terminus of Lic16A, which contains the SLH modules, was cleaved off upon expression in E. coli. The cleavage was quantitative and site-specific. A similar cleavage mechanism can reasonably be surmised for the enzyme isolated from C. thermocellum, as we find similar fragment sizes and no complete protein. A non-proteolytic, probably autocatalytic cleavage may be assumed as described for the cleavage of Asp-Pro bonds in other cellulosomal components (Lamed et al., 2001). Despite the cleavage, the SLH modules are closely attached to the rest of the protein, i.e. both parts of Lic16A co-purify in all liquid chromatography purification steps. In addition, if a His-tag is fused to the SLH module, the catalytic part of the protein is bound quantitatively to an affinity Ni-NTA column, although the His-tag peptide is not covalently connected to it. The mechanism of this phenomenon is worthwhile studying.

The expression of extra-cellulosomal Lic16A was induced by glucans containing β-1,3-linkages and its production was not growth-phase dependent. Whether the putative repressor Orf4 is involved in the regulation of expression of licA and/or other genes remains to be elucidated. The Lic16A enzyme was found to be secreted and to stay partially cell-bound, but not in the cellulosome: no activity was detected in zymograms of purified cellulosomes from strain F7 and the type strain (data not shown; Ali et al., 1995). The native protein was isolated from the cell surface with a newly described fractionation procedure using GuHCl which would also allow the isolation of other extracellular, cell-bound proteins.

C. thermocellum was described as a cellulose-hydrolysing bacterium, which grows on cellulose and cellobextrins, but not on pentoses and moreover, does not even readily grow on glucose. It was discussed that the uptake of oligo-cellobextrins is energetically favourable, because (1) energy is consumed for only one transportation event for more glucose residues, and (2) oligodextrins are degraded by intracellular cellobiose- and cellobextrin-phosphorylases producing glucose 1-phosphate (Tanaka et al., 1995; Reichenbecher et al., 1997; Lynd et al., 2002). It is not known if cellobiose or cellobextrin phosphorylases hydrolyze β-1,3-oligodextrins. However, a higher growth yield than on cellobiose or cellulose was obtained on glucans containing β-1,3-linkages, indicating that β-1,3-glucan may be phosphorolytically cleaved. β-1,3-Glucans seem to be natural substrates for C. thermocellum.

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