Nucleotide Sequence of a β-1,3-Glucanase Isoenzyme II$_A$ Gene of *Oerskovia xanthineolytica* LL G109 (*Cellulomonas cellulans*) and Initial Characterization of the Recombinant Enzyme Expressed in *Bacillus subtilis*

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The nucleotide sequence of the β*glII*$_A$ gene, encoding the extracellular β-1,3-glucanase II$_A$ (βglII$_A$) of the yeast-lytic actinomycete *Oerskovia xanthineolytica* LL G109, was determined. Sequence comparison shows that the βglII$_A$ enzyme has over 80% identity to the βglII isoenzyme, an endo-β-1,3-glucanase having low yeast-lytic activity secreted by the same bacterium. The βglII$_A$ enzyme lacks a glucan- or mannan-binding domain, such as those observed in β-1,3-glucanases and proteases having high yeast/fungus-lytic activity. It can be included in the glycosyl hydrolase family 16. Gene fusion expression in *Bacillus subtilis* DN1885 followed by preliminary characterization of the recombinant gene product indicates that βglII$_A$ has a pI of 3.8 to 4.0 and is active on both laminarin and curdlan, having an acid optimum pH activity (ca. 4.0).

Oerskovia xanthineolytica is a yeast-lytic actinomycete, now considered a subjective synonym of *Cellulomonas cellulans* (30). It was previously known as *Arthrobacter luteus*. *O. xanthineolytica* produces an extracellular lytic enzyme system containing mannanase, chitinase, protease, and glucanase activities. Endo-β-1,3-glucanases (EC 3.2.1.39 and EC 3.2.1.6) are one of the major components of such systems and have been extensively studied in a number of *O. xanthineolytica* strains (15, 19, 20, 24, 32, 33). Such enzymes are able to decompose the β-1,3-glucan component found in the cell wall of *Saccharomyces cerevisiae* and other β-1,3-glucans such as laminarin, curdlan, and pachyman. Most *Oerskovia* and closely related *Arthrobacter* strains seem to secrete into the medium multiple β-1,3-glucanase activities generally showing different substrate specificities and patterns of action (5, 15, 19, 35). Such is the case of *O. xanthineolytica* LL G109 (16); original characterization studies of the lytic enzyme system of this strain (32) revealed the presence of at least two molecular forms of glucanase, although only one of them, a 31-kDa type II β-1,3-glucanase with a pI of 4.8 to 5.0 (designated βglII) was characterized. Reexamination of the fermentation broth from strain LL G109 by isoelectric focusing followed by activity staining specific for β-1,3-glucanase has recently revealed the presence of at least two additional molecular forms of glucanase with pIs of ca. 6.0 to 6.5 and 3.8 to 4.0 (17). Frequently, only some of the observed molecular forms of β-glucanase that yeast/fungus-lytic bacteria synthesize are capable of readily solubilizing yeast glucan and inducing lysis of viable yeast cells (glucanases having high lytic activity, or type I glucanases [5]). The other type of glucanases solubilize yeast glucan only partially, causing limited cell lysis (glucanases having low lytic

activity, or type II glucanases [5]). In the case of *Bacillus circulans*, the first bacterium isolated with the ability to induce lysis of yeast and fungal cells, there is now convincing evidence that the multiplicity of lytic glucanases that a particular strain produces reflects the presence of several glucanase-encoding genes in its genome (1, 10). The genetic relationships between lytic glucanases in the genus *O. xanthineolytica* are still unclear. The number of genes encoding yeast/fungus-lytic glucanases so far cloned and sequenced from this genus is very limited. So far, every glucanase enzyme component of yeast/fungus-lytic enzyme bacterial systems whose corresponding gene has been isolated and sequenced corresponds to glucanases type I (26, 37, 38). These enzymes show a multidomain structure similar to that found in some cellulases; i.e., the catalytic domain of the enzyme is separated from the polysaccharide-binding domain(s) by linker sequences of variable length (12). In contrast, nothing is known about the structure of the putative genes coding for type II glucanases.

The present work reports the nucleotide sequence of the β*glII*$_A$ gene from *O. xanthineolytica* LL G109. This gene has been previously isolated and cloned in *Escherichia coli* by screening a partial genomic library for *O. xanthineolytica* LL G109, using a PCR-generated DNA fragment as a heterologous probe. This DNA fragment corresponded to an internal region of the putative β*glII* gene coding for the type II endo-β-1,3-glucanase II produced by the same organism (9, 32, 33). In a preliminary study (9), we analyzed chromosomal DNA from *O. xanthineolytica* LL G109 (16) by Southern blot hybridization using the 180-bp PCR-generated fragment as a probe. Multiple bands of hybridization corresponding to different *Bam*HI- and/or *Kpn*I-generated DNA fragments (e.g., >10- and 2.7-kb *Bam*HI fragments) were detected. This finding suggested the possibility of a second gene closely related to the β*glII* gene, namely, β*glII*$_A$, also being present in the *O. xanthineolytica* LL G109 genome. First, we decided to clone and identify a smaller 2.7-kb *Bam*HI-generated DNA fragment from strain LL G109 corresponding to one of the bands of

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Description and/or relevant genotype[a]	Source or reference
Strains		
E. coli JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*	39
B. subtilis DN1885	*amyE*	3
Plasmids (size in kb)		
pUC18 (2.7)	Ap^r, pBR322 origin of replication	Pharmacia
pPF8A (5.4)	pUC18 plus a 2.7-kb *Bam*HI fragment from LL G109 genomic DNA containing β*glII*$_A$ ORF	9
pDN2801 (2.8)	Cm^r, pUB110 origin of replication	3
pDN520 (7.6)	Cm^r Km^r, pBD64 origin of replication, *amyM*	2

[a] Ap^r, ampicillin resistance gene; Cm^r, chloramphenicol resistance gene; Km^r, kanamycin resistance gene; *amyM*, maltogenic α-amylase gene from *B. stearothermophilus*.

hybridization. A partial genomic library of *O. xanthineolytica* LL G109 was constructed in pUC18 and transformed into *E. coli* JM109. Hybridization analysis of colony blots from this library with the PCR probe enabled us to isolate four hybridization-positive transformants. One of the isolated transformants, strain JM109 harboring plasmid pPF8A (Table 1; see Fig. 4), was used for further sequence determination. The location of the region homologous with the PCR probe was determined by Southern blot hybridization analysis to be on the 1.5-kb *Bam*HI-*Kpn*I restriction fragment contained in the 2.7-kb *Bam*HI inserts.

Sequencing of the *O. xanthineolytica* LL G109 β*glII*$_A$ gene. The nucleotide sequence of the 1.5-kb *Bam*HI-*Kpn*I fragment from the insert in pPF8A was determined directly on double-stranded plasmid DNA by the dideoxy-chain termination method (22) with a Sequenase 2.0 kit (USB-Amersham) and [^{35}S]dATP (1,000 Ci/mmol; Amersham). The DNA was additionally sequenced in the presence of 7-deaza-GTP instead of dGTP. Sequences were analyzed with the Genetics Computer Group (GCG) (11) and PCGENE (Intelligenetics Inc., Geneva, Switzerland) programs. The nucleotide sequence and corresponding predicted amino acid sequences of a 1,459-bp region of the insert in pPF8A are shown in Fig. 1. A single complete open reading frame (ORF) of 921 bp encoding a 306-amino-acid polypeptide with a predicted molecular mass of 32,741 Da was observed. This ORF was designated β*glII*$_A$ on the basis of similarity with the 180-bp PCR product described above (81.8% identity in a 180-bp overlap to a DNA region at the 3′ end of the ORF) and on the basis of similarity of its deduced amino acid sequence with partial sequences of the βglII enzyme (Fig. 1). There are three possible translation start codons in this ORF: an ATG at position 353 preceded by a potential ribosome-binding site (RBS; GGAG) 5 nucleotides upstream (starting at nucleotide 344), which resembles the consensus Shine-Dalgarno sequence GGAGG (28); an ATG one codon upstream (nucleotide 350); and a third ATG situated 11 codons upstream without any preceding Shine-Dalgarno sequence. The analysis of the sequence upstream of the putative RBS did not reveal any obvious sequences compatible with an *E. coli* type of promoter. Also, we could not find any sequence similar to putative regulatory operator-like sequences such as those found in several glucanase-encoding genes from other actinomycete strains (e.g., *Streptomyces* sp. [6]), including the β-1,3-glucanase-encoding gene from *O. xanthineolytica* ATCC 21606 (formerly *A. luteus* 73/14 and later reclassified as *C. cellulans* DSM 20424) (26) and the closely related *Arthrobacter* sp. strain YCWD3 (accession number D23668). In the 3′ flanking region, downstream from the termination codon TGA, there is an imperfect 13-bp inverted repeat sequence. However, this stem-loop structure is not fol-

lowed by a stretch of T residues, as observed in rho-independent transcription terminators. A similar feature is also found in the 3′ noncoding region of the β-1,3-glucanase gene from *O. xanthineolytica* ATCC 21606 (26). An absence of transcriptional termination and promoter-like sequences surrounding the ORF coding for βglII$_A$ indicates that the gene might form part of a cluster of ORFs of a single operon. The study of codon usage of the β*glII*$_A$ gene reveals a strong codon bias toward G and C nucleotides in the third position (GC of 85.3% in the wobble position, which reflects the 70 to 75% molar GC content of *O. xanthineolytica* DNA [31]). The β*glII*$_A$ gene shows a GC content of 67.1%. Also, Shen and coworkers (26) reported a GC content of over 72% for a 2.7-kb DNA fragment from *O. xanthineolytica* ATCC 21606 containing a β-1,3-glucanase gene.

Predicted amino acid sequence of βglII$_A$ and similarities to other proteins. Protein sequence comparison shows that βglII$_A$ has a very high similarity (81.7% identity) with available partial sequences from the βglII glucanase secreted by the same strain, a β-1,3-glucanase that has low yeast-lytic activity (type II glucanase). The NH$_2$-terminal end of the deduced amino acid sequence for βglII$_A$ exhibits signal sequence characteristics found in secreted proteins (34). Computer analysis using the PCGENE program PSIGNAL for prokaryotic secretory signal sequence processing sites predicted that the best potential cleavage site was between amino acid residues Ala-29 and Ala-30. As a result, the predicted molecular mass of the mature β-1,3-glucanase of 277 amino acid residues would be 29,855 Da. However, the NH$_2$-terminal sequence of the native mature form of the βglII enzyme purified from LL G109 fermentation broth shows a very high similarity (76% identity over 17 amino acid residues) to that predicted for the βglII$_A$ sequence at positions Thr-64 to Ser-80 (Fig. 1). This may indicate that the first 63 amino acid residues of the polypeptide are not present in the mature βglII$_A$. This region may represent a pre-pro sequence that is removed by proteolytic cleavage after residue Val-63. In this case, the molecular weight of the mature enzyme of 243 amino acid residues would be 26,480. The predicted pI for βglII$_A$ in both cases was 4.12 (calculated with the ISOELECTRIC program of the GCG package). We are presently isolating and purifying the native form of βglII$_A$ from *O. xanthineolytica* LL G109 culture supernatant in order to characterize the enzyme and elucidate the sequence of its NH$_2$-terminal end. This will help to define the beginning of the coding region and the length of the signal peptide. Comparison of the deduced amino acid sequence of βglII$_A$ with sequences in the protein database and translated nucleic acid databases by using the programs FASTA and BLAST (served through the European Bioinformatics Institute and National Center for Biotechnology Information World Wide Web servers) re-

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     BamHI
   1 ggatccggtgatgcgcgaaaccttcgtcatgaggggttcggtgcgcttgagacgcccccgc
  61 gctgtcctaccgctggtgaggacggcgcacaccgtccggtgcatcattcgggacgtcggg
 121 cccaggaggtggtcggcatcaaatcacggcatcgttcggatgacactcttcaccgttgag
                            SacI
 181 ccggggcaacaacctgtggtcctcgttcggaggagctcgtcgcagccctcgtgtcagagg
 241 tgcccgcttcgacgccggcccaggagatacgggtcgcgcagaccctcaccacgtgcgaca
                                                       BglII
 301 ccgcggacccacaccgacgatgaaggctctgccggcagatctcggagagatgatgagcct
                                             M  S  L
 361 cccgcatgagccgtcctcgccttcaaggcgaaccctcacgttgatcctggccgctgctgc
      P  H  E  P  S  S  P  S  R  R  T  L  T  L  I  L  A  A  A  A
 421 tggtctcgcactggtggccgcctggatcgtcatcgccaccaggtcgtcgccaccgacgag
      G  L  A  L  V  A  A  W  I  V  I  A  T  R  S  S  P  P  T  S
                        •
 481 tcctcccaccacagaaggcggccaggtcacgaccccagccccaacgaccccaccgccgt
      P  P  T  T  E  G  G  Q  V  T  T  P  A  P  N  D  P  T  A  V
         AvaI
 541 cacccccgagagcctcgcctggtccgacgagttcgacggcgccgcgggggtcggcgccgaa
      T  P  E  S  L  A  W  S  D  E  F  D  G  A  A  G  S  A  P  N
      A  P  G  D  L  L  W  S  D  E  F  D  G  A  A  G  S
     N-terminus βglII                                          SacI
 601 ccccgacgtgtggaaccacgagaccggcgccggcggttggggcaacgcgagctccagaa
      P  D  V  W  N  H  E  T  G  A  G  G  W  G  N  A  E  L  Q  N
 661 ctacacgacgtcgcggggtgaactcggcgctcgacggtcagggcaacctggtcatcaccgc
      Y  T  T  S  R  V  N  S  A  L  D  G  Q  G  N  L  V  I  T  A
 721 gctccaggagagcgacgggtcgtacacgtccgcacgcttgaccacgcaggtgcaacgtcca
      L  Q  E  S  D  G  S  Y  T  S  A  R  L  T  T  Q  G  N  V  Q
                                                              Y  Q
 781 gccgcagttcggtcgaatagaggcgcatccagatcccgcgtggccaggcgatctggtc
      P  Q  F  G  R  I  E  A  R  I  Q  I  P  R  G  Q  G  I  W  S
      P  Q  Y  G  R  I  E  A  R  I  Q  I  P  R  G  Q  G  I  W  P
                        Peptide 1
 841 cgcgttctggatggtcggagcgaacctgcccgacacccccctggcctacctccggtgagat
      A  F  W  M  V  G  A  N  L  P  D  T  P  W  P  T  S  G  E  I
      A  F
 901 cgacatcatggagaacgtgggcaatgcgccccacgaggtccacgcgcacggtccacgggcc
      D  I  M  E  N  V  G  N  A  P  H  E  V  H  G  T  V  H  G  P
 961 tgggtactccggggacaacggcatcatgggcacctaccagcatccgcaagggtggtcgtt
      G  Y  S  G  D  N  G  I  M  G  T  Y  Q  H  P  Q  G  W  S  F
                                    ClaI
1021 cgccgacgacttccacaccttcggcatcgattggacgccgggtgagatcacgtggctcgt
   1                                                          tttgt
      A  D  D  F  H  T  F  G  I  D  W  T  P  G  E  I  T  W  L  V
                                                               F  V
      A  V  D  W  K  P  G  E  I  T  W  F  V
                        Peptide 2
1081 tgacgggcaggagtatcaccgcgtgacgaccgcggatgtcggtgccaaccagtgggtgtt
   6 ggatgggcagcagttccaccgcgtcacgcgccgcgagctcggcgcgaacgcctgggtgtt
      D  G  Q  E  Y  H  R  V  T  T  A  D  V  G  A  N  Q  W  V  F
      D  G  Q  Q  F  H  R  V  T  R  R  E  L  G  A  N  A  W  V  F
      D  G  Q  Q  F  X  R  V
1141 cgaccagccgttcttcctcatcctcaacgtcgccatcggcggccagtggcccggcaacccc
  66 cgaccagccgttcttcctcatcctcaacgtcgtcggcgggcagtggccggctaccc
      D  Q  P  F  F  L  I  L  N  V  A  I  G  G  Q  W  P  G  N  P
      D  Q  P  F  F  L  I  L  N  V  A  V  G  G  Q  W  P  G  Y  P
                                          V  A  V  G  G  Q  W  P  G  Y  P
                        Peptide 3
1201 cgacgcaacgaccccgtttccgcagcagatgaaggtcgactacgtgcgggtctacgacaa
 126 cgacggcacgacccagctcccgcagcagatgaaggtcgattacgtccgcgtctacg
      D  A  T  T  P  F  P  Q  Q  M  K  V  D  Y  V  R  V  Y  D  N
      D  G  T  T  Q  L  P  Q  Q  M  K  V  D  Y  V  R  V  Y
                                 V  D  Y  V  R  V  Y  D
                        Peptide 4
1261 cgcgacgcagtagccaccctcgcgcggcgcgccgccggttcgatcgggaacgggtcgagac
      A  T  Q  *
1321 ctgtgggtgaacgaccagcgtcgacctgggcaacctgaccgttgctggtgcagctcg
1381 gtcatcctgtgactcctggggtctacggcggatgcgcatcgtcgctgccgcccggacgggc
1441 ggtaggggcgtcaggtacc
                   KpnI
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FIG. 1. Nucleotide sequence of βglII_A and flanking regions. The deduced amino acid sequence is shown below the DNA sequence. Restriction sites determined experimentally are shown. A potential RBS is indicated in boldface and underlined. Start and stop codons are indicated in boldface. The facing arrows at the 3′ flanking region of βglII_A mark an inverted repeat sequence. Alignment of the 180-bp PCR product nucleotide sequence (in italics) (9), containing an internal region of the putative βglII gene, is shown. The computer-predicted mature NH₂ terminus for βglII_A is indicated by a closed circle. Alignment of partial amino acid sequence from the mature NH₂ terminus of βglII and partial sequences from peptides obtained after digestion of βglII (9) are shown in boldface. Alignment of the deduced amino acid sequence of the 180-bp PCR product is shown in italics.

vealed that this sequence bears significant similarity to several endo-β-1,3-glucanases (EC 3.2.1.39), endo-β-1,3(4)-glucanases (EC 3.2.1.6), and endo-β-1,3-1,4-glucanases/lichenases (EC 3.2.1.73) from a variety of bacteria (i.e., *Rhodothermus marinus*, *Clostridium thermothellum*, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and several strains of *Bacillus* sp.). In addition, βglII_A shows significant similarity to the carboxyl-terminal region of the horseshoe crab (1→3)-β-D-glucan-sensitive coagulation factor G subunit α precursor (25). Moreover,

all of these enzymes have been classified as glycosyl hydrolases belonging to family 16 (13, 14). Thus, we have assigned both βglII_A and βglII to this family of proteins. Additional manual search and sequence comparison in the available literature also revealed a significant similarity of βglII_A with the deduced amino acid sequence, not present in the databases consulted, of a 35-kDa thermostable β-1,3-glucanase that can also be assigned to family 16, derived from an alkaliphilic *Bacillus* sp. strain, AG-430 (18). Pairwise similarity values as calculated with the BESTFIT program (GCG package) between βglII_A and all of these proteins range from 48.4 to 20.2% (percent identity over aligned sequences). These values, together with multiple alignment with the PILEUP program (GCG package) of all these sequences and βglII_A show that these enzymes can be clustered in two groups or subfamilies (Fig. 2): (i) a group of β-glucanases able to hydrolyze β-1,3-glucans or both β-1,3- and β-1,3-1,4-glucans [such is the case of the β-glucanase from *Rhodothermus marinus* (29); the coagulation factor G α subunit, a (1→3)-β-glucan-binding protein, is also included in this group] and (ii) a group of β-1,3-1,4-glucanases. βglII_A shows a higher overall percentage sequence identity with enzymes from the first group than with those from the second group. BLAST searches of PRODOM and PROSITE databases show that βglII_A has three major regions of similarity with the catalytic domains of the enzymes of this family (Fig. 2). In particular, the motif starting at position 182 of the βglII_A precursor, EIDIMENVGNAP, was found to align with the pattern PS01034 of the PROSITE database (Fig. 2). This motif corresponds to the glycosyl hydrolases family 16 active site, which has the consensus sequence E-[LIV]-D-[LIV]-X(0,1)-E-X(2,3)-G-X. Also, the amino acid motifs of βglII_A from residues 220 to 253 and from residues 146 to 177 are found to align with the β-1,3-1,4-glucanase-like domain 1457 of the PRODOM database, which is also found among family 16 β-1,3-glucanases (Fig. 2). In contrast, there is a conserved motif that appears to be characteristic of this β-1,3-glucanase group, the PRODOM domain 20584 (Fig. 2). It is interesting that conserved regions present in the enzymes in this family are fairly rich in aromatic residues, especially tryptophan. The group of β-1,3-glucanases share eight conserved tryptophan residues among them. A smaller number of tryptophan residues appear to be conserved throughout both the β-1,3-glucanases and the β-1,3-1,4-glucanases of the family. So far, there is no experimental evidence revealing a specific role for the conserved tryptophan residues in the structures and functions of these proteins (e.g., in enzyme-substrate binding). Yet it is worth mentioning the role that tryptophan residues have in substrate binding of some cellulases and other polysaccharide-binding proteins (4). Site-directed mutagenesis of these residues would be useful to demonstrate their function in endoglucanase activity and/or their substrate specificity. A number of extracellular yeast/fungus-lytic glucanases have also been purified from *B. circulans* (1, 10) and *O. xanthineolytica* (20) strains, and their NH₂-terminal sequence have been determined. The similarity of these sequences to the NH₂-terminal regions of β-1,3-glucanases from family 16 of the glycosyl hydrolases indicates that the corresponding glucanases probably belong to the same family. They also have significant similarity to the NH₂-terminal regions of βglII and βglII_A from strain LL G109 (Fig. 3). βglII_A does not show any region of significant similarity with enzyme regions identified as insoluble glucan-binding or yeast-lytic domains. Such domains have been identified in yeast and fungal enzymes with a high lytic activity such as type I lytic β-1,3-glucanases from *O. xanthineolytica* ATCC 21606 (26) and *B. circulans* WL-12 (36) or the yeast-lytic protease from *R. faecitabidus* (27). These enzymes appear to have catalytic do-

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AG430    ..............PAGNANAAPNWSLVWSDEFN...GNSLNPANWTAEIG......TGSGGWGNNELQYYT.SRPQNLQVSGGNLIITA.......QR
d16622   ..............ARICCSHEPKWQLVWSDEFT...NGI..SSDWEFEMG......NGLNGWGNNELQYY...RRENAQVEGGKLVITA.......KR
jn0772   DAPRPDVNDQEDIPIGTPNDSAIEGMNLIWQDEFN...GTALDQSKWNYETGYYLNDDPNTWGWGNSELQHYT.DRAQNVFVQDGKLNIKALNEPKSFPQ
p23903   NAPRPDVSDQEDISIGTPTDPAIAGMNLIWQDEFN...GTTLDTSKWNYETGYYLNNDPATWGWGNAELQHYT.NSTQNVYVQDGKLNIKAMNDSKSFPQ
bglIIa   PTSPPTTEGGQVTTPAPNDPTAVTPESLAWSDEFDGAAGSAPNPDVWNHETG.......AGGWGNAELQNYTTSRVNSALDGQGNLVITAL.......Q
u04836   SDKAPHWE.................LVWSDEFD..YSGLPDPEKWDYDVG........GHGWGNQELQYYTRARIENARVGGGVLIIEAR.......H
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AG430    ESYGGMNYTSARIKTQGLQSFTYGKIEARIKLPSGQGLWPAFWMLGEDPSSVG..WPYCGEIDIMERVN.NNPHVNGTVHWDA........NGHAETGRT
d16622   EDYDGFKYTSARLKTQFDKSWKYGKIEAKMAIPSFRGVWVMFWMSGDNTNYVR..WPSSGEIDFIEHRNTNNEKVRGTIHWSTP......DGAHAHHNRE
jn0772   DPSRYAQYSSGKINTKDHFSLKYGRVDFRAKLPTGNGIWPALWMLPQDNVYGT..WASSGEIDVMEAKGRLPGSTSGAVHFGGQWPTNRYLSGEYHFPEG
p23903   DPNRYAQYSSGKINTKDKLSLKYGRVDFRAKLPTGDGVWPALWMLPKDSVYGT..WAASSGEIDVMEARGRLPGSVSGTIHFGGQWPVNQSSGGDYHFPEG
bglIIa   ES..DGSYTSARLTTQGNVQPQFGRIEARIQIPRGQGIWSAFWMVGANLPDTP..WPTSGEIDIMENVGNAPHEVHGTVH.GPGYSGDNGIMGTYQHPQG
u04836   EPYEGREYTSARLVTRGKASWTYGRFEIRARLPSGRGTWPAIWMLPDRQTYGSAYWPDNGEIDIMEHVGFNPDVVHGTVH.TKAY...NHLLGTQRGGSI
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AG430    SGNLDFSQYHTYSVEWDPNYIRWFVDGVQYNE......FYIANGTGNTEEFQRPFFIILNLAVGGNWPGSPNASTPFPPAQMLVDYVRVYQDSNLSSIVN
d16622   SNTNGI.DYHIYSVEWNSSIVKWFVNGNQYFE......VKIQGGVNGKSAFRNKVFVILNMAIGGNWPGFDVADEAF.PAKMYIDYVRVYQDASTSSPVG
jn0772   QTFAN..DYHVYSVVWEEDNIKWYVDGKFFFKVTRDQWYSAAAPNNPNAPFDQPFYLIMNLAIGGTFDGGRTPDPSDIPATMQVDYVRVYKEGEGGGQNP
p23903   QTFAN..DYHVYSVVWEEDNIKWYVDGKFFYKVTNQQWYSTAAPNNPNAPFDEPFYLIMNLAVGGNFDGGRTPNASDIPATMQVDYVRVYKEQ.......
bglIIa   WSFAD..DFHTFGIDWTPGEITWLVDGQEY........HRVTTADVGANQWVFDQPFFLILNVAIGGQWPGNPD.ATTPFFQQMKVDYVRVYDNATQ.....
u04836   RVPTARTDFHVYAIEWTPEEIRWFVDDSLYYRFPN.ERLTDPEADWRHWPFDQPFHLIMNIAVGGAWGGQQGVDPEAFPAQLVVDYVRVYRWVE......
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B

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p29716   VFR...SNF.....DSVQWKK....R.WAK..FVSTVLEAFTGDI.SNGKMILTLDREYGGSYPYKSGEYRTKSFFGYGYYEVRMKAAKNVGIVSSFFTY
p37074   VF....SNF.....DSSQWEK....AD.WANGSVFNCVWKPSQVTF.SNGKMILTLDREYGGSYPYKSGEYRTKSFFGYGYYEVRMKAAKNVGIVSSFFTY
p04957   SFFDPFNGY.....NSGFWQK....ADGYSNGNMFNCTWRANNVSMTSLGEMRLALTS..PAYNKFDCGENRSVQTYGYGLYEVRMKPAKNTGIVSSFFTY
p07980   SFFEPFNSY.....NSGLWQK....ADGYSNGDMFNCTWRANNVSMTSLGEMRLALTS..PSYNKFDCGENRSVQTYGYGLYEVRMKPAKNTGIVSSFFTY
p27051   SFYEPFNNY.....NTGLWQK....ADGYSNGNMFNCTWRANNVSMTSLGEMRLSLTS..PSYNKFDCGENRSVQTYGYGLYEVNMKPAKNVGIVSSFFTY
p23904   VFWEPLSYF.....NRSTWEK....ADGYSNGGVFNCTWRANNVNFTNDGKLKLGLTS..SAYNKFDCAEYRSTNIYGYGLYEVSMKPAKNTGIVSSFFTY
p37073   AFYESFDAF.....DDERWSK...AGVWTNGQMFNATWYPEQVTADGLMRLTIAKKT..TSARNYKAGELRTNDFYHYGLFEVSMKPAKVEGTVSSFFTY
p17989   ..........................................KDFSGAELYTLEEVQYGKFEARMKMAAASGTVSSMFLY
bglIIa   AWSDEFDGAAGSAPNPDVWNHETGAGGWGNAELQNYTTSRVNSALDGQGNLVITALQESDGSYTSARLTTQGNVQPQFGRIEARIQIPRGQGIWSAFWMV
                                                                                              ProDom 1457
```

```
p29716   .TG..PSDNNPW...DEIDI.EFLGKDTTKVQFNWYKNGVGGN.....EYLHNLGFDASQDFHTYGFEWRPDYIDFYVDGKKVYRGTR....NIPVTPGE
p37074   .TG..PSDNNPW...DEIDI.EFLGKDTTKVQFNWYKNGVGGN.....EYLHNLGFDASQDFHTYGFEWRPDYIDFYVDGKKVYRGTR....NIPVTPGE
p04957   .TG..PTDGTPW...DEIDI.EFLGKDTTKVQFNYYTNGAGNH.....EKIVDLGFDAANAYHTYAFDWQPNSIKWYVDGQLKHTATN....QIPTTLGE
p07980   .TG..PTEGTPW...DEIDI.EFLGKDTTKVQFNYYTNGAGNH.....EKFADLGFDAANAYHTYAFDWQPNSIKWYVDGQLKHTATT....QIPAAPGE
p27051   .TG..PTDGTPW...DEIDI.EFLGKDTTKVQFNYYTNGVGNH.....EKIVNLGFDAANSYHTYAFDWQPNSIKWYVDGQLKHTATT....QIPQTPGE
p23904   .TG..PAHGTQW...DEIDI.EFLGKDTTKVQFNYYTNGVGGH.....EKVISLGFDASKGFHTYAFDWQPGYIKWYVDGVLKHTATA....NIPSTPGE
p37073   .TGEWDWDGDPW...DEIDI.EFLGKDTTRIQFNYFTNGVGGN.....EFYYDLGFDASESFNTYAFEWREDSITWYVNGEAVHTATE....NIPQTPGE
p17989   QNGSEIADGRPW...VEVDI.EVLGKNPGSFQSNIITGKAGAQKT..SEKHHAVSPAADQAFHTYGLEWTPNYVRWTVDGQEVRKTEGGQVSNLTGTQG.
bglIIa   GANL..PD.TPWPTSGEIDIMENVGNAPHEVHGTVHGPGYSGDNGIMGTYQHPQGWSFADDFHTFGIDWTPGEITWLVDGQEYHRVTTADVGANQWVFDQ
                       PS01034                                                                ProDom 1457
```

```
p29716   IMMNLWPGIGVDEWLGRYDG.RT.PLQAEYGICKI
p37074   IMMNLWPGIGVDEWLGRYDG.RT.PLQAEYEYVKY
p04957   IMMNLWNGTGVDEWLGSYNGVN..PLYAHYDWVRY
p07980   IMMNLWNGTGVDDWLGSYNGVN..PIYAHYDWMRY
p27051   IMMNLWNGAGVDEWLGSYNGVT..PLSRSLHWVRY
p23904   IMMNLWNGTGVDDWLGSYNGAN..PLYAEYDWVKY
p37073   IMMNLWPGVGVDGWTGVFDGDNT.PVYSYYDWVRY
p17989   LRFNLWSSES.AAWVGQFDESKL.PLFQFINWVKV
bglIIa   PFFLILNVAIGGQWPGNPDATTPFFQQMKVDYVRV
         ProDom 20584
```

FIG. 2. Multiple alignments of the deduced amino acid sequence for βglII$_A$ from strain LL G109 with β-1,3-glucanase (A), β-1,3-1,4-glucanase (B), and related sequences from several sources. Regions in βglIII$_A$ showing similarity to the pattern PS01034 from the PROSITE database, corresponding to the glycosyl hydrolases family 16 active site, and to domains 1457 and 20584 of the PRODOM database are shown in shaded boxes. Accession numbers for SWISSPROT, PIR, or GenBank databases are given as sequence abbreviations, except for the *Bacillus* strain AG-430 β-1,3-glucanase (AG430) and βglII$_A$ (boldface). Residues in the individual sequences identical to the βglII$_A$ sequence are shown in boldface.

mains separated from polysaccharide-binding domains, as found in cellulases. The ability of these enzymes to degrade insoluble glucan or lyse viable cells is reduced when these domains are removed from the catalytic domain. βglII$_A$ appears to have a significant similarity to the catalytic domain of a family 16-type I yeast-lytic β-1,3-glucanase such as glcA1 from *B. circulans* but not to its insoluble glucan-binding do-

main. This finding has led us to assume that those bacterial glucanases having a high capability to lyse yeast and fungal cells (type I) have substrate-binding domains in addition to their catalytic cores, whereas β-1,3-glucanases having low lytic activity (type II) such as βglII and βglII$_A$ do not have a domain which one could identify as a potential lytic or insoluble glucan-binding domain. βglII$_A$ does not show any significant sim-

```
βglII_A      TPESLA.WSDEFDGAAGSAPNPDVWNHETGAGGW
βglII        APGDLL.WSDEFDGAAGS
TK-1_40      APGDLL.WSDEFDGAAGSAPNPAVWNHETGAHGW
IAM1165_28   APNWNLVWXDWFNGTXLNXAN
IAM1165_42   APNXNLVWSDEFNGTXLNNA
WL-12_40     ATNWNLVWXDWFNGS
```

FIG. 3. Manual alignment of the NH_2-terminal region of βglII_A with NH_2-terminal sequences of mature β-1,3-glucanases βglII from strain LL G109, 40-kDa β-1,3-glucanase (TK-1_40) from *O. xanthineolytica* TK-1 (20), 40-kDa β-1,3-glucanase (WL-12_40) from *B. circulans* WL-12 (10), and 28- and 42-kDa β-1,3-glucanases (IAM1165_28 and IAM1165_42, respectively) from *B. circulans* IAM1165 (1).

ilarity to β-1,3-glucanases from plants such as tobacco, maize, or barley, including those which are classified among the plant pathogenesis-related proteins that can hydrolyze the (1→3)- and (1→3, 1→6)-β-glucans of fungal cell walls. The known β-1,3-glucanases from *S. cerevisiae*, which function on the yeast cell wall during morphogenetic events, do not appear to belong to family 16. Also, a pairwise alignment of βglII_A with the type I yeast-lytic 57-kDa-β-1,3-glucanase from *O. xanthineolytica* ATCC 21606 (26) reveals that although the two sequences have a 21.9% identity over aligned sequence regions, the latter does not have the conserved sequence motifs that characterize proteins from family 16. Also, the NH_2-terminal regions of βglII and βglII_A from strain LL G109 and that of the 40-kDa enzyme from strain TK-1 show no significant similarity with any region of the 57-kDa β-1,3-glucanase from *O. xanthineolytica* ATCC 21606. In fact, this enzyme still appears as unclassified in a recent update of the glycosyl hydrolase classification (13). However, it is interesting that this enzyme has 99% sequence identity with another yeast-lytic enzyme, a β-1,3-glucanase from *Arthrobacter* sp. strain YCWD3 (accession number D23668). In addition, its substrate-binding domain shows significant similarities with corresponding domains from a yeast-lytic serine protease from *R. faecitabidus* (27) and the central region of the (1,3)-β-D-glucan-sensitive coagulation factor G subunit α from horseshoe crab (25), indicating that the β-1,3-glucanase from *O. xanthineolytica* ATCC 21606 should be regarded as member of a new family of the glycosyl hydrolases.

Cloning and expression of the β*glII_A* gene in *Bacillus subtilis*. Direct expression of the β*glII_A* gene in *B. subtilis* DN1885 (3) was attempted by inserting either the 1.5-kb *Bam*HI-*Kpn*I fragment from pPF8A or the 1.1-kb *Bgl*II-*Kpn*I fragment contained in the same fragment into the *Bam*HI and *Kpn*I sites of plasmid pDN2801, producing pPF15BK and pPF11BgK, respectively (Fig. 4). In both cases, the entire ORF for β*glII_A* was under the control of the promoter for the maltogenic α-amylase from *Bacillus stearothermophilus* (2). *B. subtilis* DN1885 was subsequently transformed with these plasmid constructs by using standard procedures (40). However, neither of the two plasmids conferred β-1,3-glucanase activity on the host organism, as observed after Luria broth (LB)-laminarin plate assays, nor was βglII_A detected as a protein band after extracts from those cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses (7). Instead, we attempted to express β*glII_A* by constructing plasmid pPFF1. In this plasmid, the expression signals from the native β*glII_A* gene were replaced with those from the α-amylase-encoding gene from *B. stearothermophilus* (2) (Fig. 4). To obtain such a plasmid construct, we assumed that the NH_2 terminus of the mature native βglII_A glucanase corresponded to residue Thr-64, as deduced by similarity to the NH_2-terminal end of the βglII glucanase and not to residue Ala-30, as predicted by the PSIGNAL program. Also, the DNA region in β*glII_A* coding for Thr-64, Pro-65, and Glu-66 is overlapped by an *Ava*I restriction

site (Fig. 1). The final construct, pPFF1, was constructed as follows. A 0.2-kb *Hin*dIII-*Ava*I fragment from pPF11BgK was replaced by a 0.15-kb *Hin*dIII-*Ava*I PCR-amplified fragment, which contained the RBS and the signal peptide-encoding regions of the maltogenic α-amylase gene from *B. stearothermophilus*. The PCR fragment was obtained following pDN520 DNA amplification, using the primers pair DK15 and DK16, as follows. PCR amplification of pDN520 (2) DNA with primers DK16 (5′ GATGCAAGC**TTGCATTACGAAAGGAGAC** 3′) and DK15 (5′ CGTA<u>CTCGGGGGT</u>**GGCTTCAGCGGCGTT TGGATTGTAC** 3′) (sequences corresponding to *B. stearothermophilus* are in boldface, whereas the underlined nucleotide sequence in DK15 corresponds to the *O. xanthineolytica* LL G109 β*glII_A* gene) and *Taq* DNA polymerase was carried out by using the following standard procedures. Transformant clones harboring plasmid pPFF1 gave rise to βglII_A production in *B. subtilis* DN1885, which could be detected on LB (21) agar plates containing 0.04% (wt/vol) laminarin after 24 h of incubation at 37°C and subsequent activity staining with Congo red (23) (data not shown). Supernatants from *B. subtilis* DN1885(pPFF1) shake flask cultures were also tested for βglII_A activity on AZCL-curdlan (Megazyme, Sydney, Australia) at different pH values (4.0, 5.5, and 7.0) (8). The βglII_A relative activity on curdlan at pH 4.0 was approximately 22 times higher than the background activity levels observed with *B. subtilis* DN1885, demonstrating that *B. subtilis* actively expresses and secretes βglII_A. βglII_A relative activity in the culture supernatants was higher at pH 4.0 (22.4 times above the background activity value) and 5.5 than at pH 7.0 (14.9 and 6.4 times above the background activity value, respectively), showing that the optimum pH for activity of this enzyme must be acidic. We determined the NH_2-terminal sequence of the heterologous βglII_A product purified from 11 of fermentation broth of *B. subtilis* DN1885(pPFF1) (8), using an Applied Biosystems 473A protein sequencer (data not shown), and found it to be correctly processed by the host strain and in agreement with that predicted from the fused β*glII_A* gene construct. Isoelectric focusing analysis of the culture supernatants from DN1885/pPFF1 showed that the recombinant βglII_A corresponds to a protein band with a pI between 3.5 and 4.0 (data not shown), which is similar to that previously predicted for the amino acid sequence deduced from the fused β*glII_A* gene construct. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry of the purified recombinant βglII_A carried out in a VG Analytical TofSpec (VG Analytical, Manchester, England) indicated that it had a molecular mass of 26,462 Da, in agreement with that calculated from the amino acid sequence deduced for the fused gene product (26,480 Da) and in reasonable accordance with the mass of 30 kDa for heterologous mature protein observed on SDS-PAGE of DN1885/pPFF1 culture supernatants (data not shown). Initial characterization of the βglII_A reveals that this enzyme has a molecular weight very similar to that of βglII, but the two proteins differ in pI. Also, they have a very high degree of sequence similarity (over 80% identity). Thus, we predict that the two enzymes have very similar patterns of action and substrate specificity and can be considered isoenzymes. Yet initial characterization of the β-1,3-glucanase activity of βglII_A indicates that its optimum activity pH is clearly acidic, whereas that of βglII ranges between 6.0 and 8.0, depending on the substrate (33).

Multiple β-1,3-glucanase-encoding genes in *O. xanthineolytica* LL G109. The results described above indicate that *O. xanthineolytica* LL G109 has at least two genes coding for β-1,3-glucanases. This finding provides the first evidence at the
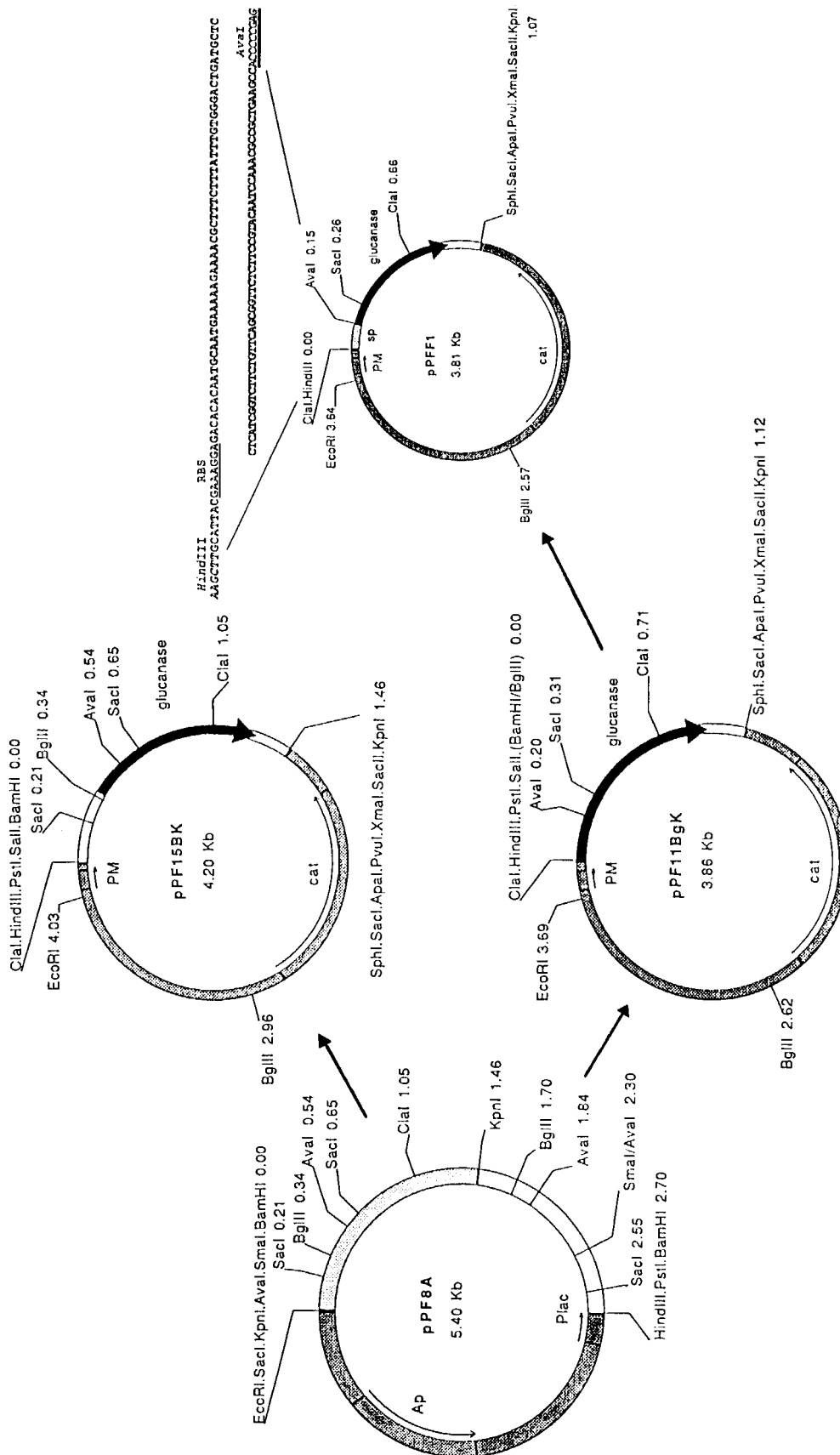
FIG. 4. Plasmids pPF8A, pPF15BK, pPF11BgK, and pPFF1. *Bam*HI-*Kpn*I-digested pDN2801 was ligated to the 1.5-kb *Bam*HI-*Kpn*I *O. xanthineolytica* LL G109 DNA fragment from pPF8A (shown in light shading) to form pPF15BK. pPF11BgK was constructed by ligating *Bam*HI-*Kpn*I-digested pDN2801 to 1.1-kb *Bgl*II-*Kpn*I-digested *O. xanthineolytica* LL G109 DNA fragment from pPF8A. Plasmid pPF11BgK was digested with *Ava*I and *Hind*III to remove the 0.2-kb fragment containing the 5′ flanking region of $\beta glII_A$. Then a 150-bp PCR fragment (sequence shown), containing the region for the RBS and the coding region for the signal peptide of the maltogenic α-amylase gene from *B. stearothermophilus*, was inserted into the *Hind*III and *Ava*I sites of pPF11BgK. Plac, *lac* promoter; PM, promoter from the maltogenic α-amylase gene from *B. stearothermophilus*; Ap, ampicillin resistance gene; cat, chloramphenicol resistance gene.

genetic level of the multiplicity of molecular species of extracellular β-1,3-glucanases being produced by strain LL G109.

Nucleotide sequence accession number. The nucleotide and deduced amino acid sequences reported here have been submitted to the GenBank database (accession number U56935).

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