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Cloning and DNA Sequencing of *bgaA*, a Gene Encoding an Endo-β-1,3-1,4-Glucanase, from an Alkalophilic *Bacillus* Strain (N137)

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The gene *bgaA* encoding an alkaline endo-β-1,3-1,4-glucanase (lichenase) from an alkalophilic *Bacillus* sp. strain N137, isolated in our laboratory, was cloned and expressed from its own promoter in *Escherichia coli*. The nucleotide sequence of a 1,416-bp DNA fragment containing *bgaA* was determined and revealed an open reading frame of 828 nucleotides. The deduced protein sequence consists of 276 amino acids and has a 31-amino-acid putative signal peptide which is functional in *E. coli*, in which the BgaA protein is located mainly in the periplasmic space. The lichenase activity of BgaA is stable between pH 6 and 12, it shows optimal activity at a temperature between 60 and 70°C, and it retains 65% of its activity after incubation at 70°C for 1 h. This protein is similar to some other lichenases from *Bacillus* species such as *B. amyloliquefaciens*, *B. brevis*, *B. licheniformis*, *B. macerans*, *B. polymyxa*, and *B. subtilis*. However, it has a lysine-rich region at the carboxy terminus which is not found in any other published lichenase sequence and might be implicated in the unusual biochemical properties of this enzyme. The location of the mRNA 5′ end was determined by primer extension and corresponds to nucleotide 235. A typical *Bacillus* σ^A promoter precedes the transcription start site.

Members of the genus *Bacillus* produce and secrete into culture media a broad variety of hydrolytic enzymes with activity against several complex substrates. The industrial interest for members of this genus is well illustrated in the production of enzymes such as α-amylase, proteases, glucanases, glucose isomerase, and restriction endonucleases, etc. (30, 36). Among these enzymes, endo β-(1,3)-(1,4)-glucanases (lichenases) have specific applications in the brewing industry in that their activity facilitates the access of the α-amylases to the starch of the endosperm cells, facilitating the filtration process and reducing gels and haze formation in the finished products (1). Lichenases also have applications in poultry foodstuffs to improve the β-glucan digestibility (10). These enzymes, which hydrolyze (1-4)-β linkages in 3-O-substituted β-D-glucanopyranosyl residues, are unable to hydrolyze repeating sequences of β-1,3- or β-1,4-linked glucans such as laminarin or carboxymethylcellulose (CMC), respectively. Genes encoding these carbohydrolases have been cloned from different species of *Bacillus* such as *B. subtilis* (8), *B. amyloliquefaciens* (4), *B. macerans* (6), *B. circulans* (7), *B. polymyxa* (13), *B. licheniformis* (21), and *B. brevis* (21a), and most of these genes have been cloned and expressed in heterologous hosts such as *Escherichia coli*, *B. subtilis*, and yeasts (7, 9, 12, 15). Several hybrid enzymes have been obtained from *B. amyloliquefaciens* and *B. macerans* endo β-(1,3)-(1,4)-glucanases by using the PCR and extension of several overlapping segments. Some of the hybrid enzymes showed a significant increase in thermostability when exposed to an acidic environment (28).

Here, we describe the cloning and sequencing of an endo-β-1,3-1,4-glucanase gene from an alkalophilic strain, *Bacillus* sp. strain N137. This gene is expressed from its own promoter in *E. coli* in which the protein BgaA is located mainly in the periplasmic space. The BgaA protein possesses sequences similar to those of endo-β-1,3-1,4-glucanases from other *Bacillus* species but has a lysine-rich COOH terminus which is not present in any other glucanases. BgaA is active even at pH 12, maintaining 80% of the activity that is optimal at pH 7.5, and is unusually stable to elevated temperatures. Other studied *Bacillus* lichenases normally have an acidic or neutral optimal pH and are not stable to high pHs and temperatures. These differential characteristics make the BgaA protein a good candidate for industrial use.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The *Bacillus* strain used in this work was N137 (a strain isolated as an alkaline cellulase producer by C. Tabernero and J. Sánchez [39a]). The *E. coli* strains used were MV1190 (Bio-Rad) for single-strand DNA propagation and DH5α (14) and MC 1061 (25) for subcloning and plasmid isolation. The plasmid vectors used were pIJ2925 (a pUC18 derivative [19a]) and pBluescript KS+ and KS− (Stratagene). The plasmids derived from this work are indicated in Fig. 1.

Media and culture conditions. PY medium (11) was used for glucanase and xylanase production by the *Bacillus* sp. All liquid cultures were carried out in 250-ml flasks with 50 ml of medium. Cultures were incubated at 37°C at 250 rpm in an orbital shaker (Adolf Kühner AG Schmeiz) for 72 h. *E. coli* strains were cultivated in Luria-Bertani (LB) medium and 2× YT (34) and transformed by the Hanahan method (14). Selective antibiotic media containing either ampicillin (50 μg/ml) or kanamycin (50 μg/ml) were used when needed.

Enzyme preparation and gel electrophoresis. *Bacillus* sp. strain N137 culture supernatants were separated from cellular mass by centrifugation at 10,000 × *g* for 10 min. The culture supernatant was used as a crude enzyme preparation to

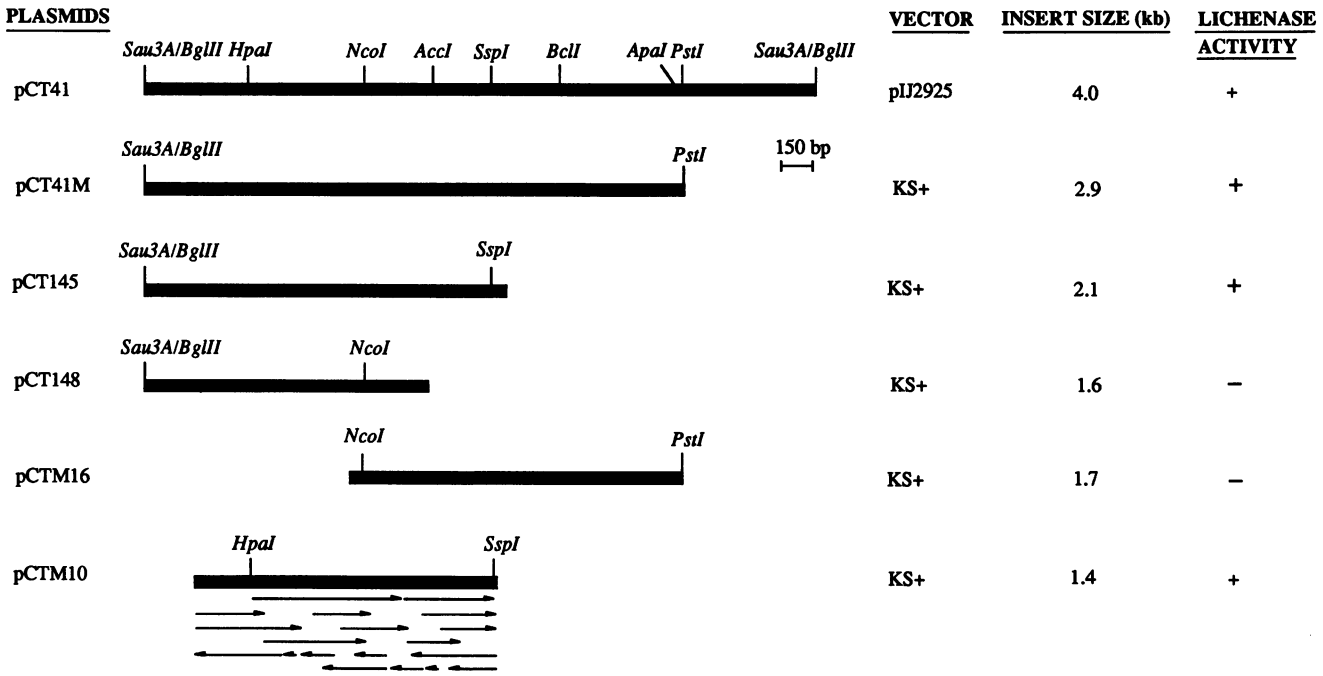

FIG. 1. Plasmids used during this work. Only the restriction enzymes used for subcloning in the nucleotide sequence determination are shown. Plasmid names are indicated on the left. The vectors used (pIJ2925 and pbluescript KS+), the size of the DNA inserts and the lichenase activity (with [+] or without [–]) are indicated on the right. Arrows, below pCTM10, indicate the regions and directions of sequencing.

quantify the glucanase activity. For denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were precipitated with 10% trichloroacetic acid, resuspended in loading buffer (20), boiled for 5 min, and run on 12% polyacrylamide gels. The gels were run according to the method of Laemmli (20) in a Mini Protean II system (Bio-Rad). After SDS-PAGE, the proteins present in the gels were stained with Coomassie blue, and their sizes were estimated by using Bio-Rad low-molecular-weight markers. Lichenase activity was detected in agarose gels containing 0.1% lichenan by the zymogram technique of Béguin (2).

Obtention of proteins from *E. coli*. *E. coli* cells were grown in LB at 37°C overnight and harvested by centrifugation at 7,000 × *g* for 10 min. Total extracts were obtained as follows: the cells were resuspended at 0.5 g/ml (wet weight) in 20 mM phosphate buffer (pH 8.0), and then the extracts were obtained in a French press at 16,000 lb/in^2. The cell extract was treated for 45 min at 37°C with DNase I and RNase A and then centrifuged at 30,000 × *g* for 40 min. The supernatant was used as an enzymatic solution or loaded onto polyacrylamide gels.

To locate the BgaA protein in *E. coli*, cells containing the *bgaA* gene were fractionated into culture supernatant and periplasmic and cytoplasmic fractions, as described by Sambrook et al. (33). Those fractions were used as crude enzyme, and the lichenase activity was assayed in all of them. Alkaline phosphatase and β-galactosidase were used as periplasmic and cytoplasmic enzyme markers, respectively.

Effects of pH and temperature on lichenase activity and stability. Lichenase activity was measured in *E. coli* cell extracts and *Bacillus* sp. strain N137 culture supernatants at various pHs (from pH 5 to 12) by using lichenan as the substrate. The buffers used were sodium acetate (pH 5), phosphate buffer (pH 5.5 to 8), glycine-NaOH (pH 8 to 10),

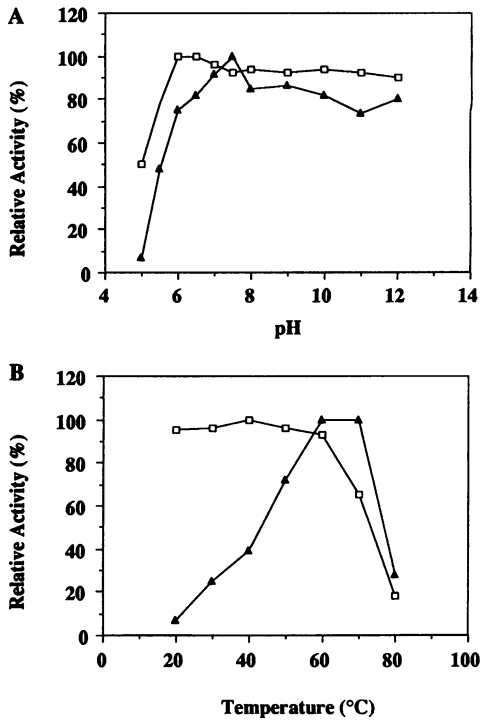

FIG. 2. Effect of pH (A) and temperature (B) on activity (▲) and stability (□) of BgaA. The buffers used were sodium acetate at pH 5, phosphate at pH 5.5 to 8, glycine-NaOH at pH 8 to 10, and glycine-NaOH-NaCl at pH 10 to 12. The final concentration of all the buffers in the reaction mixture was 200 mM.

and glycine-NaOH-NaCl (pH 10 to 12), each at a concentration of 200 mM in the reaction mixture. To evaluate the stability of this crude enzyme at several pHs, the enzyme preparation was preincubated for 1 h at different pHs without the substrate, and the activity was assayed later at pH 9.

The optimum temperature of the crude enzyme was determined by varying the incubation temperature at pH 9. The stability at different temperatures was determined at pH 9 for 1 h, and later the activity was assayed at 60°C.

Enzyme assays. Lichenase activity was determined as the production of reducing sugars by the dinitrosalicylic acid colorimetric assay, using glucose as the standard (26). One lichenase unit was defined as the release of 1 μmol of glucose equivalents per min under the standard assay conditions (100 μl of enzyme solution and 1% lichenan in a 400-μl final assay volume of 0.2 M glycine-NaOH buffer [pH 9] incubated at 70°C for 10 min).

The protein concentration was estimated by the method of Lowry et al. (22), with bovine serum albumin as the standard.

Recombinant DNA techniques. Total DNA was isolated from strain N137 as described previously for *Streptomyces* spp. (18). *E. coli* plasmids were isolated as described by Birnboim and Doly (3). T4 DNA ligase, restriction endonucleases, and modifying enzymes were purchased from Amersham, Bethesda Research Laboratories, and Boehringer-Mannheim Biochemicals and used following the manufacturers' guidelines and standard protocols (33).

Construction of a genomic library of *Bacillus* sp. strain N137 in the plasmid pIJ2925 and selection of carbohydratase-positive clones. Total DNA (10 μg) isolated from strain N137 was partially digested with *Sau*3AI and size fractionated in an 0.8% agarose gel. Fragments with sizes between 2 and 5 kb were purified by electroelution (33) and ligated to the *Bam*HI-digested and dephosphorylated pIJ2925. The ligation mixtures were used to transform *E. coli* MC1061, and transformants were selected on plates of LB agar medium containing 50 μg of ampicillin per ml. The transformants were counted, harvested by the addition of 5 ml of sterile water, and stored in 20% glycerol at −80°C until used for the screening.

DNA sequencing. Overlapping deletions were generated on plasmid pCTM10 with exonuclease III as described by Henikoff (16). After subcloning in Bluescript KS+ and SK+ plasmids (Stratagene), fragments with appropriate sizes were sequenced in both strands by the dideoxy chain termination method (34), with the T7 Sequencing Kit (Pharmacia LKB). DNA and protein sequence analyses were done with the DNASIS, PROSIS (Pharmacia-LKB and Hitachi), and DNA Strider (23) programs. The deduced amino acid sequence of BgaA was compared with sequences in the SWISS PROT Data Bank with the FASTA programs (29). Alignments of protein sequences were performed with the CLUSTAL program (17).

RNA isolation. *Bacillus* cells were grown in 200 ml of PY liquid medium with or without 1% lichenan (Sigma) as a carbon source. The cells from a 20-ml aliquot were harvested by centrifugation (10,000 × *g* for 20 min) at 12, 24, 36, 48, and 72 h. The RNA was prepared as described by Igo and Losick (19), but the amounts of all the reagents were scaled for 25-ml cultures. The final RNA pellet, after DNase treatment, was dissolved in diethyl pyrocarbonate-treated water, quantified by spectrophotometry, and stored at −70°C until used.

For slot blot, 0.2 μg of RNA was redissolved in 200 μl of sample buffer (10 mM phosphate buffer [pH 7], 6.6% glyoxal), applied to the Zeta-Probe membrane, and transferred as recommended by the manufacturer (Bio-Rad).

Prehybridization was carried out for 4 to 6 h at 50°C with constant agitation in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide–50 mM phosphate buffer (pH 7)–5× Denhardt's solution (33)–1% SDS–50 μg of heat-denatured salmon sperm DNA per ml. The hybridization was done overnight at 50°C in an identical solution but with a nick-translated, α-^{32}P-labeled *bgaA* probe containing the 1,250-bp *Hpa*I-*Ssp*I fragment of *bgaA* (Fig. 1) and 10% dextran sulfate. Blots were washed three times for 10 min in 2× SSC at room temperature and later for 35 min in 2× SSC–0.1% SDS.

The slot blot-exposed films were scanned with the Bio-Image System (Millipore).

Nucleotide sequence accession number. The sequence of *bgaA* reported in this paper has been submitted to the EMBL data base under accession no. Z12151.

RESULTS

Isolation of N137. Strain N137 was selected after the screening of about 4,000 isolated colonies from alkaline soil for their capacity to hydrolyze different polymers of carbohydrates under alkaline conditions. This strain grows optimally at 37°C and pH 9. Several attempts were made to identify *Bacillus* sp. N137 at the species level, but it was not possible to include it in any known *Bacillus* species (39a).

Cloning of *Bacillus* sp. strain N137 carbohydratase genes in *E. coli*. A *Bacillus* sp. strain N137 library was made in *E. coli* MC 1061 with the pIJ2925 plasmid. To select for clones producing different glycanases, transformants were plated in LB medium supplemented with 0.5% CMC, xylan, laminarin, or lichenan (depending on the type of enzyme to be detected) and incubated at 37°C for 48 h. The plates were then stained with Congo red (40), and colonies surrounded by a clear halo were selected and studied in more detail. Among 11,120 transformants, not one overproduced β-1,4-endoglucanases. However, one clone encoding a xylanase, two clones encoding laminarinases, and five clones encoding lichenases were selected.

From this point our work focused on the plasmids present in the lichenase overproducer clones. Restriction patterns showed that all five plasmids carried a similar DNA fragment. These plasmids were named pCT41 (Fig. 1), pCT61, pCT102, pCT151, and pCT211 (data not shown). Derivative plasmids were constructed from plasmid pCT41 by using restriction enzymes and/or exo III deletions. The lichenase activity was assigned to a 1.4-kb DNA fragment present in the plasmid pCTM10 (Fig. 1). The presence of the cloned DNA fragment in *Bacillus* sp. strain N137 was confirmed by Southern hybridization analysis.

Cellular location of the BgaA protein in *E. coli*. *E. coli* carrying plasmid pTCM10 was grown in LB medium for 16 h and fractionated as described by Sambrook et al. (33). Enzymatic activity was determined in the cytoplasmic and periplasmic fractions and in the extracellular medium. β-Galactosidase and alkaline phosphatase were used as cytoplasmic and periplasmic markers, respectively. The lichenase activity was found mainly (80%) in the periplasmic fraction, indicating that the signal peptide of BgaA is used by the secretion system of *E. coli*. Moreover, the lichenase activity in *E. coli* was independent of the DNA fragment orientation. This result suggests that the DNA insert carries its own promoter, which is functional in *E. coli*.

Enzymatic properties of cloned BgaA. The enzymatic properties of BgaA were examined in extracts of *E. coli* harboring pCTM10 at different pHs and temperatures. As shown in Fig. 2A, the crude enzyme is active over a broad pH range (from pH 6 to 12). It has optimal activity at pH 7.5 and retains more

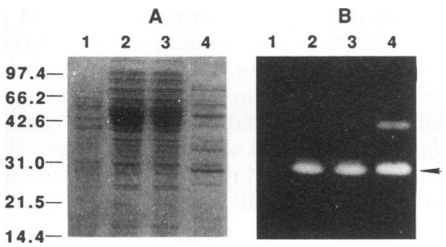

FIG. 3. SDS-PAGE of *E. coli* lysates and *Bacillus* sp. strain N137 supernatant. (A) Protein stained by Coomassie brilliant blue R-250; (B) BgaA activity, on renatured gels, detected by the zymogram technique of Béguin (2). Lanes: 1, *E. coli* MC 1061; 2, *E. coli* MC 1061 carrying pCT41; 3, *E. coli* MC 1061 carrying pCTM10; 4, *Bacillus* sp. strain N137. The arrowhead indicates the position of the protein BgaA. Molecular mass markers (in kilodaltons) are indicated on the left.

than 80% of the activity to pH 12. The stability at several pHs was studied by incubation of the same *E. coli* extract at different pHs without substrate for 1 h, and the residual activity was determined at pH 9. This enzyme is stable between pH 6 and 12 for at least 1 h. The activity of the crude extract was also determined at different temperatures. The optimum temperature for this enzyme is between 60 and 70°C, and it is almost inactive at 20°C (Fig. 2B). Crude enzyme thermostability was also assayed between 20 and 80°C for 1 h. The enzyme retains 65 and 20% of its activity after incubations at 70 and 80°C, respectively.

Substrate specificity. The enzymatic activities of the *E. coli* extracts carrying pCTM10 were assayed using CMC, xylan, laminarin, and lichenan as substrates and analyzed by the dinitrosalicylic acid method. No activity was detected against CMC, xylan, and laminarin, whereas up to 15 U/mg of protein was detected against lichenan. This was corroborated by zymogram analysis on agarose gels containing 0.5% CMC or lichenan. No band of activity appeared when CMC was used (data not shown), but a clear band (at approximately 29 kDa) of lichenase activity was detected (Fig. 3), indicating that BgaA is specific for this substrate. Culture supernatant from N137, used as a control, showed the same 29-kDa band and a faint 40-kDa band. This 40-kDa band corresponds to a cellulase with activity also against lichenan (Fig. 3B, lane 4) (39).

Nucleotide sequence analysis. The nucleotide sequence of the DNA insert carried by plasmid pCTM10 was determined in both strands by the dideoxynucleotide chain termination method (34). The 1,416-bp nucleotide sequence is shown in Fig. 4. Only one open reading frame of 828 nucleotides was

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   1  ACTCAGTTGTAATTAAGCGGATTCAATTATGTAACTCATGTTTATAAAAACATAATTGAGCCTTACTTTTCCTATTAATCTTACGATACTGTTTTGCTGA

 101  AGTCTAAAATAGAATAGGATAAGAAAAGTTTGTTAGTTTTAAATTATAACTAAATTAATTGGCAGTTTGTGAATAAGTAAACTTTATTAATTAAATTATT
        -35                -10        *
 201  GTAGTTTATTTCCAGTTGTTATATAATTCTAAATGTAAGCGGATACAACAACTTAGAATAGTTAACAAATAGAAAATTAATAAAAGTTAGGAGATGGTTC

   1                                                                                    M  K  K  K  L
 301  TTTCACTTAATTTATCTATTCTACGAGATTGGCCTAGTTTTTTTTTGAAATTAATTAACGCGCGTTAGTAGGGAGGGAGAAGGATTAATGAAAAAGAAAT

   6   V  L  F  S  M  C  L  L  L  F  S  G  L  I  S  G  L  V  Q  S  P  Q  V  A  E  A  A  E  R  P  I  G  T
 401  TAGTTTTATTTTCTATGTGTTTATTATTGTTTAGCGGTCTTATATCTGGATTAGTTCAAAGTCCACAAGTTGCTGAAGCAGCAGAAAGACCAATTGGGAC

  39   A  F  V  E  T  F  E  S  Y  D  S  E  R  W  S  K  A  G  V  W  S  N  G  Q  M  F  N  A  T  W  Y  P  E
 501  TGCATTTGTCGAAACATTTGAATCTTATGACTCAGAACGTTGGTCCAAAGCGGGAGTTTGGTCAAATGGACAAATGTTTAATGCGACATGGTATCCAGAA

  72   Q  V  T  F  S  N  G  K  M  K  L  Q  I  E  K  E  D  N  E  T  A  S  P  P  Y  K  A  G  E  L  R  T  N  D
 601  CAAGTTACTTTTTCTAATGGTAAGATGAAGTTACAAATTGAAAAAGAAGACAATGAAACTGCAAGCCCGCCATACAAAGCTGGAGAACTTCGCACAAACG

 106   F  Y  H  Y  G  L  F  E  V  S  M  K  P  A  K  S  T  G  T  V  S  S  F  F  T  Y  T  G  P  W  D  W  E
 701  ATTTTTATCATTACGGGTTGTTTGAAGTGAGTATGAAACCTGCAAAGTCAACGGGAACAGTCTCTTCATTTTTCACCTATACTGGACCTTGGGATTGGGA

 139   N  D  P  W  D  E  I  D  I  E  F  L  G  K  D  T  T  K  I  Q  F  N  Y  F  T  N  G  V  G  G  N  E  H
 801  GAATGATCCATGGGATGAAATTGATATCGAATTTTTAGGTAAGGATACTACTAAAATACAATTTAATTATTTTACAAACGGAGTAGGCGGAAATGAGCAT

 172   Y  H  E  L  G  F  D  A  A  D  D  F  N  T  Y  A  F  E  W  R  P  E  S  I  R  W  F  V  N  G  E  L  V  H
 901  TACCATGAATTAGGATTTGATGCAGCAGATGATTTTAATACGTATGCTTTTGAGTGGAGACCAGAATCTATTCGTTGGTTTGTAAATGGTGAATTGGTTC

 206   T  A  T  E  N  I  P  Q  T  P  Q  K  I  M  M  N  L  W  P  G  I  G  V  D  G  W  T  G  R  F  N  G  E
1001  ATACAGCAACAGAAAATATACCACAAACCCCACAAAAAATAATGATGAACTTATGGCCTGGTATTGGAGTAGATGGATGGACTGGTAGATTTAATGGAGA

 239   D  T  P  V  V  T  Q  Y  D  W  V  K  Y  T  P  L  E  E  L  G  C  Y  N  E  K  N  N  K  Y  K  K  C  K
1101  AGATACTCCTGTAGTTACACAGTACGATTGGGTGAAGTATACACCACTTGAGGAACTGGGCTGTTACAATGAGAAAAATAATAAATACAAGAAATGTAAG

 272   K  T  K  V  K  *
1201  AAAACGAAGGTAAAATAGTAAATCAATTGATGAAGTATTGTGAAACATGACAGATAATAATGAGGTTGTCGCAATGGGTAAACACTTTGTGGCAACCTTT

1301  TTATTAAGAAATCAATTGACTTTTACCATATCATTTCTTTGGAATGGAAAAATCAGAACATTCTTTAATAATCACGCAATCATACTAAAATAATTGCTAT
          SspI
1401  AATAAGGGAAAATATT
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FIG. 4. Nucleotide and deduced amino acid sequence of *Bacillus* sp. strain N137 *bgaA* gene and its flanking regions. The −35 and −10 sequences are located upstream from the transcriptional start point, which was determined by primer extension (asterisk). The putative −35 and −10 regions are shown in bold type. Facing arrows at the end of the *bgaA* sequence mark an inverted repeat sequence. The putative mature N terminus is indicated by a vertical arrow; this sequence is preceded by a signal peptide of 31 amino acids.

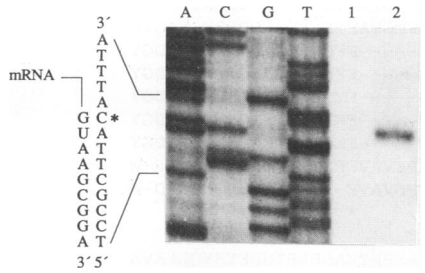

FIG. 5. Primer extension analysis to determine the transcriptional start point for *bgaA* in *Bacillus* sp. strain N137. The hybridization temperature was 37°C, and the method used was that described by Sambrook et al. (33). The sequence of the nucleotide used was complementary to the sequence between nucleotides 471 and 491 (Fig. 4). This oligonucleotide was end labeled with [γ-^{32}P]ATP. The DNA sequence ladder belongs to the *bgaA* gene obtained by the sequencing method of Sanger et al. (34) and the same synthetic oligonucleotide. The sequence of the ladder is written at the left. The transcription start point is indicated by an asterisk and the 5′ end of the mRNA is also shown. Lanes: 1, RNA from *E. coli* carrying pBluescript (negative control); 2, RNA from strain N137.

found. The open reading frame starts in ATG and encodes a protein of 276 amino acids that has a putative signal peptide at its NH_2 terminus. Comparison with other cleavage sites recognized by signal peptidase suggested that the putative cleavage might occur between two alanine residues at positions 31 and 32. The predicted size of the mature protein (28.1 kDa) is in full agreement with the molecular mass of the enzyme determined by SDS-PAGE. The codon usage is similar to that of other *Bacillus* lichenase genes. The noncoding regions flanking this gene are enriched in A+T residues (73%) in contrast to the translated sequence that has an A+T content of 63%. Downstream from the translation stop codon (TAG), there is an imperfect 20-nucleotide inverted repeat which resembles a *rho*-independent transcription termination signal (32) with a $\Delta G°$ of -21 kcal (ca. -88 kJ)/mol. Preceding the start codon there is a potential ribosome binding site (Shine-Dalgarno sequence), 5′-AGGGAGAAG-3′, which has seven matches of 9 residues with the 3′-end *B. subtilis* and *E. coli* 16 rRNA (24, 38).

Mapping the 5′ terminus of *bgaA* mRNA. The transcriptional start point was determined by primer extension with the oligonucleotide 3′-CGACTTCGTCGTCTTTCTGG-5′, which is complementary to the nucleotides between 471 and 491, as the primer. The 5′ end of the mRNA was identified as nucleotide 235 (Fig. 5). The putative promoter regions (-35 [TTGTAG] and -10 [TATAAT] [Fig. 4]) highly resemble σ^A *Bacillus* promoters (27). The sequence shows an AT-rich region upstream of the promoter that is typical for strong *B. subtilis* promoters (31).

Size of the *bgaA* mRNA transcripts and effect of the carbon source. The length of the *bgaA* mRNA transcript was characterized by Northern (RNA) blot analysis using total RNA from *Bacillus* sp. strain N137 and *E. coli* carrying pCT41. In both, the transcripts are approximately 1.3 kb long, indicating that this gene is expressed monocistronically from its own promoter (data not shown).

The effect of the carbon source on the abundance of the *bgaA* mRNA transcript was studied on slot blots of total N137 RNA obtained at different times from cultures on PY medium or in PY with 1% lichenan. The results (Fig. 6A) demonstrated that lichenan induced the appearance of *bgaA* mRNA at 12 h,

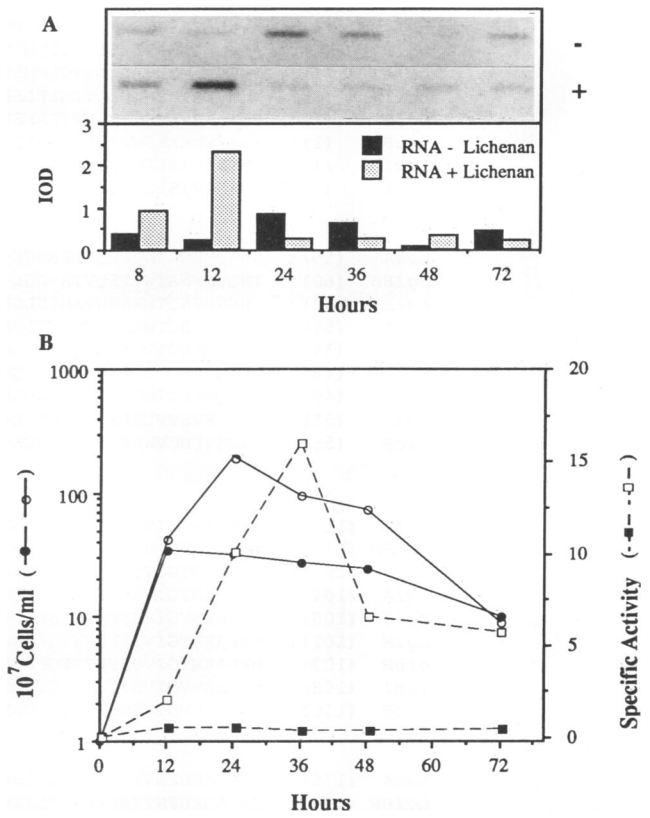

FIG. 6. Effect of lichenan present in the growing medium over the *bgaA* mRNA level (A) and on the production of protein BgaA by N137 (B). (A) Slot blot of 0.2 μg of total mRNA transferred to Zeta-Probe membranes and hybridized with the nick-translated *Hpa*I-*Ssp*I fragment of *bgaA*. The mRNA was obtained, at the time indicated, from medium with (+) or without (−) 1% lichenan. Histogram of the *bgaA* mRNA level analyzed with a Bioimage System from Millipore. IOD, integrated optical density. (B) Lichenase production (squares) and cell growth (circles) (10^7 cells per ml) in the presence (open symbols) and absence (closed symbols) of lichenan.

at which time the amount of this mRNA was at least 10 times that in the cultures without lichenan.

The lichenan induction was also detected in the assay of the lichenase activity present in N137 culture supernatants. In this case, maximum induction was reached at 36 h, at which time the activity in cultures with lichenan was 48 times higher than in cultures without it (Fig. 6B).

Similarity of the amino acid sequence of BgaA protein to those of other proteins. The predicted amino acid sequence of the protein BgaA was compared with other protein sequences in the SWISS PROT data bank (release 22) by using the CLUSTAL program. A high degree of similarity was found with lichenases from mesophilic and thermophilic species of *Bacillus* and lichenases from *Clostridium thermocellum* (Fig. 7). The similarity between these proteins is stronger in the central and C-terminal parts of the proteins where there are fragments with 100% identity. However, BgaA has a stretch of 22 residues that are lysine rich (8 amino acids) at its COOH terminus that does not exist in any other lichenase sequence. This region has a theoretical isoelectric point of 10.7 in contrast to the total protein that has a calculated pI of 4.79. The effect of this region with the unusual biochemical properties of this enzyme is currently being studied.

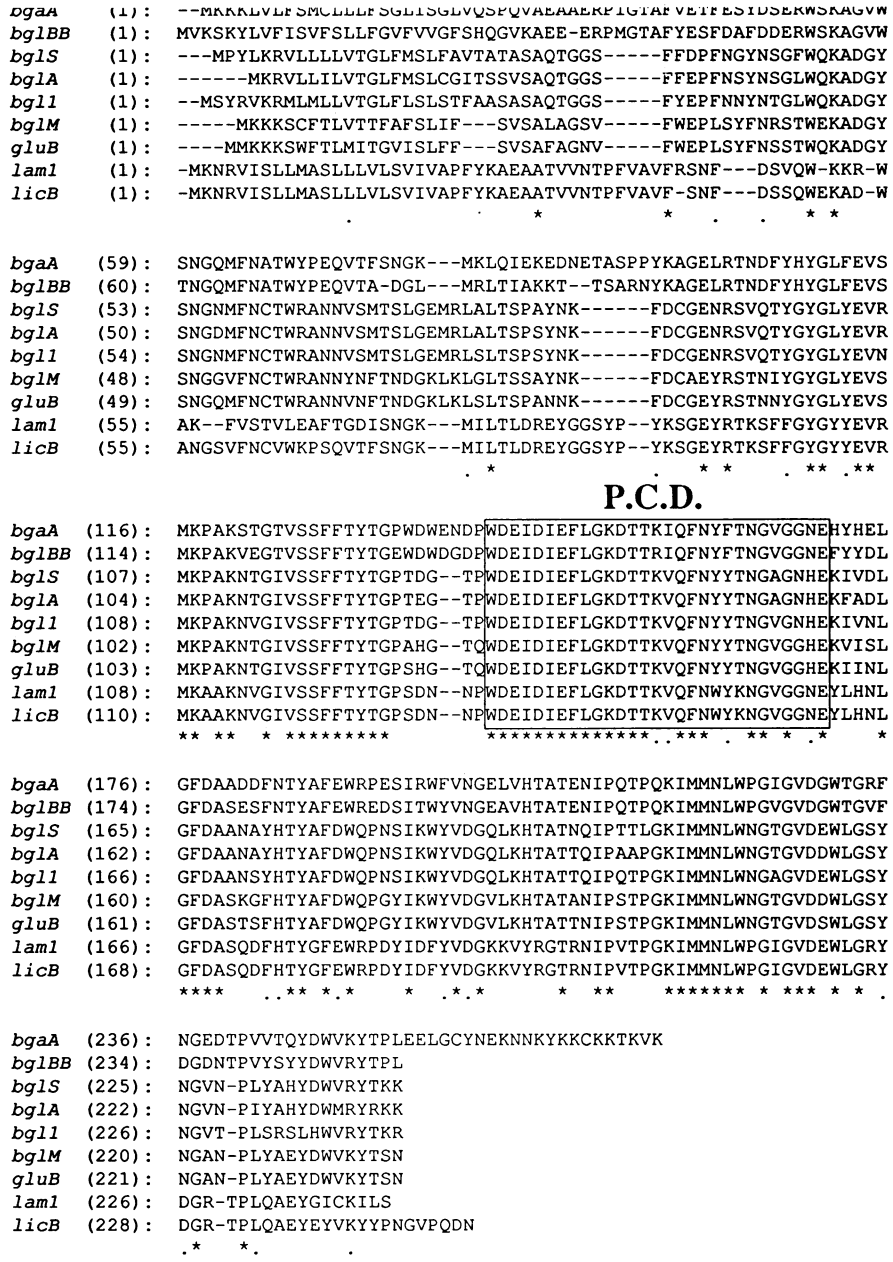

FIG. 7. Amino acid alignment, using the CLUSTAL program, of sequences belonging to several β-1,3-1,4-glucanases (lichenases). Numbering of the amino acids includes the signal peptide. P.C.D., proposed catalytic domain (boxed). The introduction of gaps to improve the alignments is indicated by dashes. Asterisks and dots below the alignment lines indicate identical and conserved amino acids residues, respectively, among all these lichenases. The following genes from the species indicated were used: *bgaA*, *Bacillus* sp. strain N137; *bglBB*, *B. brevis*; *bglS*, *B. subtilis*; *bglA*, *B. amyloliquefaciens*; *bglL*, *B. licheniformis*; *bglM*, *B. macerans*; *gluB*, *B. polymyxa*; and *laml* and *licB*, *C. thermocellum* (35, 41). Only the catalytic domain is shown in the protein originated from *licB*.

DISCUSSION

The industrial use of alkaline enzymes has attracted the interest of many research groups during the past few years. Our group has isolated several mesophilic alkalophilic *Bacillus* spp. on the basis of their capacity to produce enzymes that hydrolyze carbohydrates under alkaline conditions. In this paper we deal with the cloning and sequencing of the gene *bgaA* which encodes an alkaline β-1,3-1,4-glucanase. The biochemical properties of BgaA are different from those of other published *Bacillus* lichenases. Like lichenase from *Bacillus* sp. strain KSM 64 (37), it has high activity at pH 9. While the lichenase from *Bacillus* sp. strain KSM 64 is inactive at pH 11, BgaA retains more than 80% of the activity even at pH 12. Like *B. macerans* lichenase, BgaA has optimum activity between 60 and 70°C; however, *B. macerans* lichenase is almost inactive after incubations of 10 min at 65°C while BgaA retains

at least 65% of its activity after incubation at 70°C for 1 h. All these properties make the protein BgaA a good candidate for use in industrial processes.

Two bands of activity on lichenan were detected in the zymogram analyses of supernatants of strain N137 (Fig. 3B); one of these bands corresponds to the protein BgaA (approximately 29 kDa) and the other corresponds to a protein with a molecular mass of 40 kDa, which is an alkaline cellulase that also has activity against lichenan. This strain produces at least one other cellulase with a high molecular weight that is not detected under the conditions used (39).

The BgaA signal peptide is functional in *E. coli* in which up to 80% of the active protein is located in the periplasmic space. Similar results have been reported for the secretion of several β-glucanases from different species of *Bacillus* expressed in *E. coli* in which 60% of these proteins were found in the periplasm and extracellular fluid (5, 21).

At 24 and 36 h, cultures of N137 grown in PY liquid medium without lichenan showed a small increase in the amount of the *bgaA* transcript without a parallel increase in the lichenase activity present in the culture supernatant. A possible explanation of this result could be deduced if the efficient translation of *bgaA* mRNA needs a lichenan-dependent translational activator. In this case, if lichenan is present, the activator is synthesized and translation of *bgaA* mRNA occurs; however, if lichenan is absent, the activator is absent, too, and the translation of this gene is not possible. The low sensitivity of the activity detection method (dinitrosalicylic acid method) must be considered, too.

Comparison of BgaA with other related enzymes such as β-1,4-glucanases and β-1,3-glucanases did not reveal any clear homology, suggesting a different origin for these enzymes. The proposed catalytic domain (Fig. 7) has homology to the catalytic domain of T4 lysozyme as described by Borris et al. (5) and Lloberas et al. (21) for the β-glucanase of *B. macerans*, *B. licheniformis*, and other *Bacillus* spp. BgaA has homology to the catalytic residues Glu and Asp present in the domain WDE-X7-KDT-X2/6-YYT from T4 lysozyme, where X is one amino acid and the numbers indicate the number of residues. This domain includes the motif W-X3-BIZ-X3K (where B and Z represent Asx and Glx, respectively) present in most of the bacterial lichenases (13). This supports the hypothesis that the endo-β-1,3-1,4-glucanases might act by an acid catalysis mechanism as do other endo- and exo-glucosidases (5).

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