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Cloning and Characterization of Two Genes from *Bacillus polymyxa* Expressing β-Glucosidase Activity in *Escherichia coli*

L. GONZÁLEZ-CANDELAS, M. C. ARISTOY, J. POLAINA,* AND A. FLORS

Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas, Jaime Roig 11, 46010 Valencia, Spain

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DNA fragments from *Bacillus polymyxa* which encode β-glucosidase activity were cloned in *Escherichia coli* by selection of yellow transformants able to hydrolyze the artificial chromogenic substrate *p*-nitrophenyl-β-D-glucopyranoside. Restriction endonuclease maps and Southern analysis of the cloned fragments showed the existence of two different genes. Expression of either one of these genes allowed growth of *E. coli* in minimal medium with cellobiose as the only carbon source. One of the two enzymes was found in the periplasm of *E. coli*, hydrolyzed arylglucosides more actively than cellobiose, and rendered glucose as the only product upon cellobiose hydrolysis. The other enzyme was located in the cytoplasm, was more active toward cellobiose, and hydrolyzed this disaccharide, yielding glucose and another, unidentified compound, probably a phosphorylated sugar.

The name β-glucosidase appears in the literature as a rather undefined term for enzymes able to hydrolyze cellobiose (*O*-β-D-glucopyranosyl-1,4-β-D-glucopyranoside) and chemically related glucosides. Together with endo- and exoglucanases, some of these enzymes play an essential role in the biological saccharification of cellulose (23). β-Glucosidases are common in microorganisms, even among species unable to degrade cellulose, and they have been purified from many different sources. Genes that code for these enzymes have been cloned from several species of bacteria (3, 4, 6, 25) and fungi (7, 17, 19).

β-Glucosidases have different mechanisms of action. Hydrolysis of cellobiose may occur by cleavage of the glucosidic bond to yield two molecules of glucose. This reaction is catalyzed by the enzyme cellobiase (β-D-glucoside glucohydrolase, EC 3.2.1.21), which is present in bacteria and yeasts (6, 19). A second mechanism consists of P_i-dependent phosphorolysis of cellobiose into one molecule of glucose and one molecule of glucose-1-phosphate. This reaction is catalyzed by cellobiose phosphorylase (EC 2.4.1.20), an enzyme which has been described in bacteria (2, 3, 21) and fungi (5). Finally, a different mechanism of phosphorolysis which yields glucose and glucose-6-phosphate has been described (15).

β-Glucosidases show activity against different glucosides. In addition to cellobiose and cellodextrins, other substrates used in the characterization of these enzymes are arylglucosides (such as arbutin, salicin, and the chromogenic compound *p*-nitrophenyl-β-D-glucopyranoside), alkylglucosides (methyl-β-D-glucoside), β-1,3-glucosides (laminaribiose), β-1,2-glucosides (sophorose), etc. With the patterns of specificity observed, it is difficult to establish the natural substrates of some of these enzymes and their in vivo roles.

In this report, we describe the cloning of two genes that code for different β-glucosidases from *Bacillus polymyxa* and the characterization of the enzymes encoded by these genes expressed in *Escherichia coli*.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. polymyxa* ATCC 842 was used as the source of DNA for cloning the genes. Plasmid pUC13 (24) was used as the cloning vector, and *E. coli* DH5α (F⁻ *endA1 hsdR17 gyrA96 thi-1 recA1 relA1 supE44* φ80d *lacZΔM15*) was used as the host strain.

Cloning procedures and gene characterization. Total DNA from *B. polymyxa* and plasmid DNA from *E. coli* were purified by standard procedures (9, 10). The DNA from *B. polymyxa* was partially digested with endonuclease *Sau*3AI and electrophoresed in a 0.7% agarose gel from which fragments with sizes ranging between 3 and 10 kilobases were purified by electroelution in a Unidirectional Electro-eluter-Analytical Electroelution System (IBI). Portions of the recovered DNA were mixed with approximately equal amounts of pUC13 previously cut with *Bam*HI and dephosphorylated with calf alkaline phosphatase to a final DNA concentration of about 20 μg/ml. These mixtures were incubated overnight at 4°C with T4 DNA ligase and then used to transform competent *E. coli* cells to ampicillin resistance. A total of 3,000 such transformants containing random inserts of the *B. polymyxa* chromosome were kept in a collection. Recombinant clones from this genomic library expressing β-glucosidase genes were detected on plates of complete medium (LB) supplemented with ampicillin, overlaid with 0.7% agar containing 200 μg of the chromogenic compound *p*-nitrophenyl-β-D-glucopyranoside (PNPG) per ml in 50 mM citrate-phosphate (CP) buffer, pH 7.0.

Southern blots and nick translation were performed by standard procedures (9). Hybridizations were performed overnight at 42°C in a solution containing 50% formamide. The filters were washed at 65°C for 15 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer, for 30 min in 2× SSC containing 0.1% sodium dodecyl sulfate, and for 10 min in 0.1× SSC.

Assays of enzyme activity. β-Glucosidase activity was assayed in 50 mM CP buffer, pH 7.0, at 37°C with PNPG as the substrate at a final concentration of 5 mM. *p*-Nitrophenol released from the reaction was measured in a spectrophotometer at 400 nm. Substrates other than PNPG were used at the same concentration, except for carboxymethyl cellulose, barley β-glucan, and laminarin, which were used at 0.2%.

Glucose was measured with a 510A glucose assay kit (Sigma Chemical Co.). Protein concentration was determined with the Bio-Rad Laboratories assay by measuring the A_{595} with bovine serum albumin as the standard.

To investigate the localization in *E. coli* of the enzymes encoded by the cloned genes, periplasmic activity was assayed by using osmotically shocked cells prepared by the method of Neu and Heppel (13). Intracellular activity was measured in the cytoplasm of the same cells subjected to osmotic shock and subsequently disrupted by sonication. Alkaline phosphatase activity, assayed as described by Walter and Schütt (26), was monitored in these experiments as a control of periplasmic activity.

HPLC characterization of enzyme products. Reaction mixtures consisting of crude extracts of *E. coli* transformants (300 μg of protein per ml) with cellobiose as the substrate (8.5 mg/ml) were incubated for 1 h at 37°C and then injected into a high-pressure liquid chromatograph equipped with an Aminex HPX-87H ion-exclusion column (Bio-Rad). The column was operated at 40°C and eluted with 0.08 N H_2SO_4 at a flow rate of 0.5 ml/min. Sugars formed as products of β-glucosidase activity were monitored with a refractive index detector.

RESULTS

Cloning and physical characterization of genes. *E. coli* transformants carrying *B. polymyxa* DNA inserts in pUC13 were tested for β-glucosidase activity by the plate assay with PNPG as described in Materials and Methods. Six yellow (positive) clones were found among 3,000 colonies screened. Restriction endonuclease mapping of the plasmids carried by the clones revealed that three of them were identical. Figure 1 shows the maps of the four different plasmids characterized (pBG1, pBG2, pBG3, and pBG4) and the subclones derived from them. Two clones, those harboring pBG3 and pBG4, showed considerably higher PNPGase activity than the other two, as revealed by the intensity of the yellow in the plate assay. A variation of this assay, using the fluorescent compound methylumbelliferyl-β-D-glucopyranoside instead of PNPG allowed semiquantitative evaluation of the enzymatic activity based on the sizes of the halos surrounding the colonies under UV illumination.

Figure 2 shows the results of a Southern analysis performed to test the identities of pBG plasmids. Three different probes were used. A labeled fragment including most of the insert of pBG1 showed strong hybridization with the plasmid from which it was derived and also with pBG2 (tracks 1 and 4 in Fig. 2A) but none at all with the other two pBG plasmids or with pUC13, which was used as a negative control. An identical pattern was observed when the insert of pBG2 was used as a probe (Fig. 2C). Plasmids pBG3 and pBG4 gave a strong signal when the insert of pBG4 was used as a probe (tracks 2 and 3 in Fig. 2B). These results indicate the existence in *B. polymyxa* of two different genes that encode β-glucosidases, one of them contained in pBG1 and pBG2, the two clones showing weaker PNPGase activity, and the other one contained in pBG3 and pBG4, the clones with stronger activity. Henceforth, these genes will be designated *bglA* and *bglB*, respectively.

Localization of enzyme activities expressed in *E. coli*. Cultures of *E. coli* transformants carrying plasmids with either one of the two cloned genes were subjected to osmotic shock by the procedure cited in Materials and Methods. β-Glucosidase activity was assayed in the periplasmic fraction, in the intracellular fraction, and in the cell debris with PNPG as the substrate. The periplasmic enzyme alkaline phosphatase was also assayed in the different fractions as a control. The results (Table 1) showed that the enzyme encoded by *bglA* was located in the cytoplasm, while the one encoded by *bglB* was found in the periplasm.

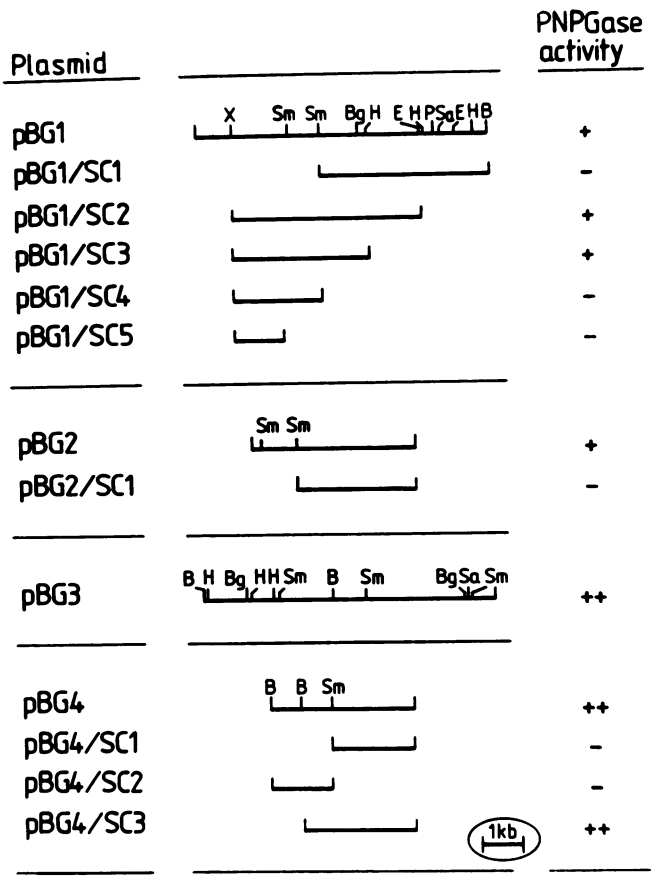

FIG. 1. Restriction maps of the insertions carried by four original *bgl* plasmids which confer β-glucosidase activity on *E. coli* and the subclones derived from them. Enzymes used were the following: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Sa, *Sac*I; Sm, *Sma*I; X, *Xba*I. Different levels of enzyme activity are represented by + and ++. kb, Kilobase.

Growth of *E. coli* in minimal medium with cellobiose as the carbon source. Expression of either one of the two cloned *bgl* genes conferred on *E. coli* the ability to grow in minimal medium with cellobiose as the sole carbon source. *E. coli* cultures harboring pBG2 or pBG4 were inoculated in minimal A medium (11) with 0.2% cellobiose as the carbon source and supplemented with 50 μg of ampicillin per ml. In different repetitions of the experiment, both cultures showed slow but remarkable growth, whereas a third one of a clone carrying pUC13, used as a control, grew not at all (Fig. 3).

Substrate specificity. *E. coli* cultures carrying either pBG2 or pBG4 were grown to the early stationary phase in minimal A medium with cellobiose as the carbon source and supplemented with ampicillin. Crude extracts prepared from these cultures were assayed with different glucosidic substrates (Table 2). The enzymes encoded by *bglA* and *bglB* were both active on cellobiose and PNPG, but the PNPGase activity of the second one was about 10 times higher. These enzymes also showed activity toward other glucosides, such as *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-D-galactopyranoside, and salicin. A control culture carrying pUC13,

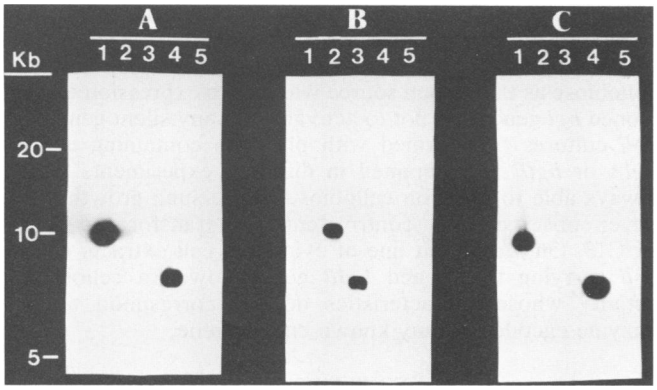

FIG. 2. Southern hybridization analysis of pBG plasmids. Autoradiograms of three filters designated A, B, and C are shown. In all three filters, the disposition of samples is as follows: track 1, pBG1; track 2, pBG3; track 3, pBG4; track 4, pBG2; track 5, pUC13 (negative control). All of the samples were linearized with *SalI*, which has a restriction site within the pUC13 polylinker but cuts none of the inserts. Filter A was probed with the *Bam*HI-*Xba*I fragment covering most of the pBG1 insert. Filter B was probed with the pBG4 insert. Filter C was probed with the pBG2 insert (Fig. 1). Kb, Kilobases.

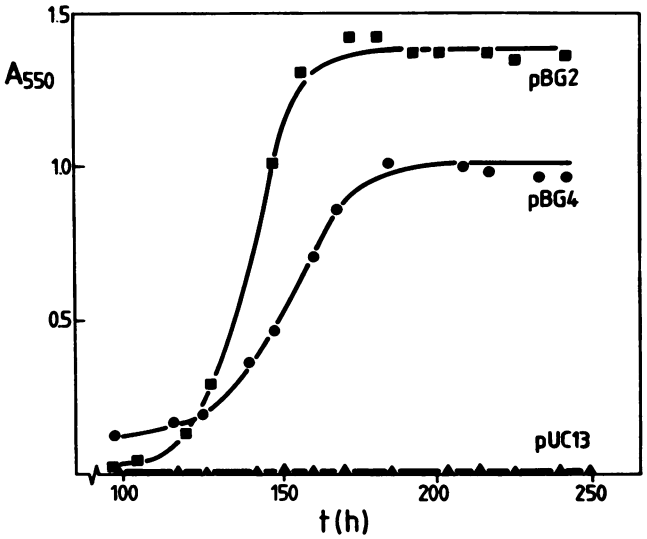

FIG. 3. Growth curves of *E. coli* transformants in minimal medium with cellobiose as the carbon source. Cultures of clones carrying plasmids pBG2 and pBG4 with the cloned genes *bglA* and *bglB*, respectively, and a control culture carrying cloning vector pUC13 were shaken at 37°C in liquid minimal A medium with 0.2% cellobiose and 50 μg of ampicillin per ml. Samples from the culture were taken at 8- to 12-h intervals, and growth was monitored by measuring the A_{550}. Contamination tests were carried out along with the experiment with negative results.

grown with 0.2% glucose instead of cellobiose, showed no significant activity toward any of the substrates tested.

Mechanisms of enzyme activity. The mechanism of action of the enzymes synthetized in *E. coli* from the two cloned genes was investigated by HPLC analysis of the products formed after incubation of crude preparations of the enzymes with cellobiose as the substrate. The chromatograms (Fig. 4) showed that the enzyme encoded by *bglA* hydrolyzed cellobiose, producing approximately equal amounts of glucose and another, unidentified compound. This compound, possibly a phosphorylated sugar, did not seem to be glucose-1-phosphate or glucose-6-phosphate, as indicated by its position in the chromatogram (peak 2 in Fig. 4C), slightly delayed in relation to that corresponding to phosphorylated glucose (peak 1 in Fig. 4B). Moreover, the presence of glucose phosphate in this reaction was not detected either by enzymatic determination (data not shown). Results obtained with the product of the *bglB* gene showed that this enzyme yielded only glucose (Fig. 4D).

DISCUSSION

Our results show the existence in *B. polymyxa* of two genes that code for different β-glucosidases. One of these enzymes, the one encoded by the gene that we have designated *bglB*, is probably an extracellular protein, because the product synthesized in *E. coli* from this gene was located in the periplasm. Genes from gram-positive bacteria that code for extracellular enzymes in their natural hosts are, in most cases, found in the periplasm when cloned in *E. coli* (8, 12, 14, 18, 27). Although the enzyme encoded by *bglB* was able to hydrolyze cellobiose, yielding glucose as the only product, the natural substrate for this enzyme could be any glucoside other than cellobiose, as suggested by its relatively low activity on cellobiose and its high activity toward artificial arylglucosides, such as PNPG, *p*-nitrophenyl-β-D-cellobioside, and *p*-nitrophenyl-β-D-galactopyranoside. This

TABLE 1. Localization of enzyme activity in *E. coli* transformants carrying plasmids pBG2 and pBG4

Fraction tested	pBG2 (*bglA*)		pBG4 (*bglB*)	
	% β-Glucosidase	% AP[a]	% β-Glucosidase	% AP[a]
Periplasm	9.2	87.2	90.3	92.2
Cytoplasm	83.5	7.9	9.3	5.0
Cell debris	7.3	4.9	0.4	2.8

[a] AP, Alkaline phosphatase.

TABLE 2. Enzymatic activity in crude extracts prepared from *E. coli* transformants carrying pBG plasmids with different glucosides as substrates[a]

Substrate	Sp act (mU)	
	pBG2 (*bglA*)	pBG4 (*bglB*)
Cellobiose	658	227
PNPG	364	3,495
PNPC	12	267
PNPX	11	11
PNPGal	72	144
Sucrose	26	23
Salicin	46	120
Maltose	41	12
Lactose	37	9
Gentibiose	19	23
Laminarin	11	4
CMC	3	0
Barley β-glucan	8	1

[a] One unit of enzyme activity was defined as the amount of enzyme which released 1 μmol of *p*-nitrophenol (for PNPG, PNPC, PNPX, and PNPGal) or 1 μmol of glucose (for the other substrates) per min. Abbreviations: PNPG, *p*-nitrophenyl-β-D-glucopyranoside; PNPC, *p*-nitrophenyl-β-D-cellobioside; PNPX, *p*-nitrophenyl-β-D-xylopyranoside; PNPGal, *p*-nitrophenyl-β-D-galactopyranoside; CMC, carboxymethyl cellulose.

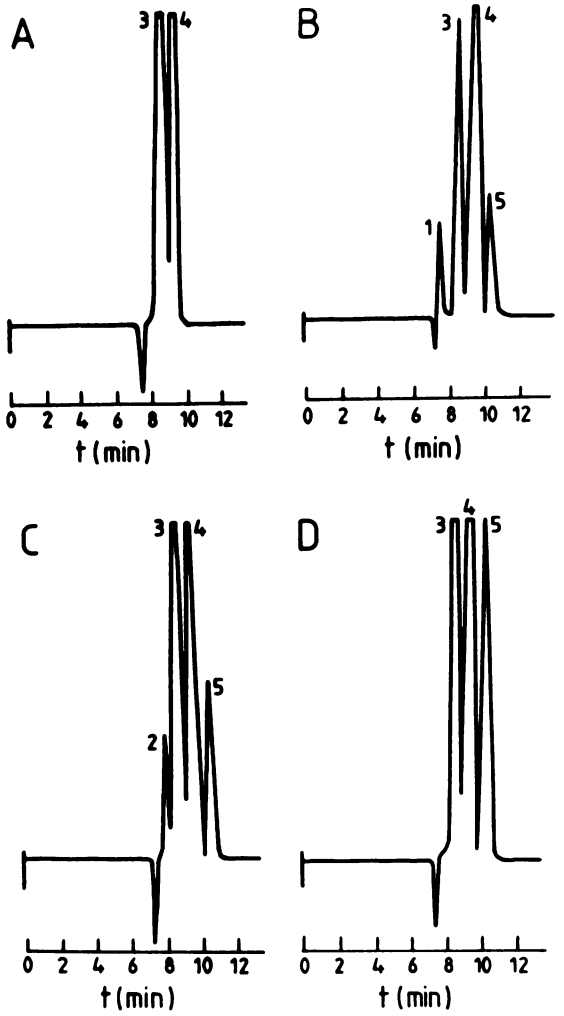

FIG. 4. HPLC analysis of products of cellobiose hydrolysis by *bgl*-encoded enzymes. (A) Control experiment with 8.5 mg of cellobiose per ml (peak 3) in 50 mM CP buffer, pH 7.0 (peak 4). (B) Control experiment with 0.5 mg of glucose-1-phosphate per ml, 0.5 mg of glucose-6-phosphate per ml (both unresolved in peak 1), 1 mg of cellobiose per ml (peak 3), and 1 mg of glucose per ml (peak 5) in 50 mM CP buffer, pH 7.0 (peak 4). Glucose, glucose-1-phosphate, and glucose-6-phosphate were included in this experiment to characterize the elution profile of probable products of cellobiose hydrolysis. (C) Profile of a reaction mixture of a crude extract of *E. coli* expressing *bglA* incubated with 8.5 mg of cellobiose per ml in 50 mM CP buffer, pH 7.0. (D) The same as for panel C but with *bglB*.

enzyme resembles very much the one from *Clostridium thermocellum* described by Aït et al. (1).

The other enzyme, encoded by the gene designated *bglA*, showed relatively higher activity on cellobiose and lower activity on other glucosides. Hydrolysis of cellobiose by crude extracts of *E. coli* expressing the gene yielded glucose and another, unidentified product which is possibly a phosphorylated sugar. Thus, this enzyme might be an intracellular cellobiose phosphorylase similar to that described by Alexander for *C. thermocellum* (2).

Expression of either *bglA* or *bglB* supports the growth of *E. coli* in minimal medium with cellobiose as the sole carbon source. Wild-type strains of *E. coli* are natural mutants unable to utilize β-glucosides although they have cryptic genes that give them the potential to do so. Mutations in four different loci have been reported which implement this capability (16, 20, 22). Nevertheless, our results indicate that growth of *E. coli* transformants in minimal medium with cellobiose as the carbon source was due to expression of the cloned *bgl* genes and not to activation of any silent gene. *E. coli* cultures transformed with plasmids containing either *bglA* or *bglB* and obtained in different experiments were always able to grow on cellobiose, while such growth was never observed for control cultures transformed with pUC13. On a different line of evidence, cell extracts of *E. coli* carrying the cloned *bglB* gene showed a cellobiase activity whose characteristics do not correspond to an enzyme encoded by any known cryptic gene.

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