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Synthesis and Secretion of a *Bacillus circulans* WL-12 1,3-1,4-β-D-Glucanase in *Escherichia coli*

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The synthesis and secretion of a 1,3-1,4-β-D-glucanase were studied in different strains of *Escherichia coli* transformed with plasmids carrying the *Bacillus circulans* WL-12 1,3-1,4-β-D-glucanase structural gene. This gene (named *BGC*) is contained within a 1.9-kilobase *Bam*HI-*Hin*dIII fragment and directs the synthesis in *E. coli* of an enzyme that specifically degrades lichenan. Only one active form of the enzyme was found when the gene was expressed in different *E. coli* strains. The electrophoretic pattern of this protein showed a molecular weight that was approximately the same as that of the mature β-glucanase secreted from *B. circulans* WL-12, suggesting that the processing of this protein may be similar in both species. As deduced from maxicell experiments, the *Bacillus* parental promoter directs the synthesis in *E. coli*. Pulse-chase experiments showed that the protein may be cotranslationally processed.

Species of the genus *Bacillus* synthesize a broad variety of extracellular hydrolytic enzymes with different activities, some of which have been extensively studied in view of their commercial importance (28). The advent of genetic engineering technology has focused interest on the production of such enzymes. On one hand, the characterization of the genetic systems controlling their synthesis may represent a crucial step for increasing their yield for industrial purposes. On the other hand, knowledge of the mechanisms of secretion of these enzymes may contribute to a much better understanding of the phenomenon of protein export in gram-positive bacteria in general and the use of bacilli as hosts for the secretion and efficient synthesis of foreign proteins in particular.

Exocellular β-glucanases are produced by many members of the *Bacillaceae* family. Among them, some act as β-glucan-endohydrolases, hydrolyzing mixed-linked β-glucans containing 1,3-β- and 1,4-β- linkages, such as lichenan and barley β-glucan. The fact that these bacterial enzymes possess a substrate range similar to that of the lichenase of germinating barley has increased their potential value for the brewing industry as substitutes and supplements of malt enzymes to hydrolyze barley β-glucans during the brewing process. Genes coding for this kind of carbohydrolase have been cloned from different species of *Bacillus* such as *B. subtilis* and *B. amyloliquefaciens*; some of these genes have also been sequenced.

Bacillus circulans WL-12 is a gram-positive microorganism isolated by Tanaka and Phaff (36) from soil samples and characterized by its ability to lyse a number of fungal and yeast cell walls. This organism synthesizes a number of carbohydrolases such as α-glucanases, chitinases, β-xylanases, and β-glucanases (13). Among the latter, 1,3-β-glucanases and 1,6-β-glucanases have been characterized (7, 35). We have detected in culture supernatants of this microorganism an endo-1,3-1,4-β-glucanase, which is not produced until the stationary phase of growth. In the present work we describe the cloning and expression of this *B. circulans* β-glucanase gene in *Escherichia coli*. The gene product is completely secreted from the cytoplasm to the periplasmic space but only partially liberated from this space to the surrounding medium.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Bacillus* strains used were *B. circulans* WL-12 (prototroph [36]), *B. circulans* NRRL-NRS 397 (prototroph), *B. subtilis* Marburg 168 (*trpC2*), *B. polymyxa* NCTC 10343 (prototroph), *B. coagulans* 26 (prototroph), and *B. amyloliquefaciens* NCIB 10785 (prototroph). The host strains of *E. coli* were C600, DH1, HB101 (17), and CSR603 (31). The *E. coli* plasmid vector was pBR322 (3).

Culture media and growth conditions. *B. circulans* WL-12 was maintained and cultured as previously described (8) in a growth medium, buffered at pH 6.5 with 0.1 M sodium phosphate buffer, containing 0.7% yeast nitrogen base and 0.5% glucose. *E. coli* strains were routinely grown in L-broth (LB) medium (6). For plasmid amplification, substrate specificity studies, and maxicell experiments, M9 minimal medium and M9CA medium (17) were also used. In cellular fractionation studies, the strains were cultured in low-phosphate medium (26) supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside to induce β-galactosidase activity. When necessary, selective antibiotic media contained ampicillin (50 μg/ml) or tetracycline (10 μg/ml). For in situ enzyme assays, the media were supplemented with 0.3% lichenan (1,3-1,4-β-D-glucan from *Cetraria islandica*).

Detection and assay for β-glucanase activity. Bacterial colonies were tested for β-glucanase activity by the Congo red screening method of Cantwell and McConnell (5). After the colonies had grown on LB-agar medium containing lichenan, plates were flooded with Congo red dye (0.1% in 100 mM Tris hydrochloride [pH 8]); the β-glucanase producing colonies were surrounded by a clear halo against a red-stained agar. Extracellular, periplasmic, and cellular

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enzyme fractions were assayed for the ability to release reducing sugar groups from lichenan. Dialyzed samples (0.1 ml) were added to an equal volume of the substrate (0.5% lichenan) in 50 mM acetate buffer (pH 5.5). The mixtures were incubated at 30°C for various periods of time, and the reducing power released was determined by the method of Somogyi (33) and Nelson (25). The results were referred to glucose as a standard; 1 U of activity was defined as the amount of enzyme that catalyzed the release of reducing sugar groups equivalent to 1 nM of glucose per h under the reaction conditions described above. When necessary, samples were assayed in a similar way for 1,4-β-D-, 1,3-β-D-, or 1,6-β-D-glucanase activity with carboxymethyl cellulose, laminarin (1,3-β-glucan from *Laminaria hyperborea*), or pustulan (1,6-β-glucan from *Umbilicaria pustulata*), respectively, as the reaction substrate.

Isolation, restriction, and ligation of DNA. Chromosomal DNA from *B. circulans* WL-12 was isolated by cesium chloride-ethidium bromide gradient centrifugation of cleared cell lysates obtained by the method of Marmur (18). For restriction analysis and transformations, plasmid DNA was prepared by the extraction procedure of Birnboim and Doly (2), for rapid small scale preparations, or by the method of Godson and Vapnek (9), with CsCl-ethidium bromide centrifugation, for larger preparations.

Digestion of DNA with various restriction endonucleases was performed under the conditions described by the suppliers (Boehringer Mannheim Biochemicals; Bethesda Research Laboratories, Inc.; and New England BioLabs, Inc.). Ligation of endonuclease-generated fragments to yield recombinant plasmids was carried out by using T4 DNA ligase for at least 12 h at 15°C in 66 mM Tris hydrochloride (pH 7.4)–6.6 mM $MgCl_2$–10 mM dithiothreitol–2 mM ATP.

Gel electrophoresis. DNA fragments produced by endonuclease digestion were analyzed by electrophoresis on agarose gels (between 0.6 and 1.2% agarose). When necessary, fragments were recovered by the electroelution method or by using low-melting-point agarose to separate them, followed by passage through Elutip columns (Schleicher & Schuell, Inc.).

Separation of proteins was performed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Laemmli (16) and by Hames (10); gels were stained for protein with silver nitrate (22). Occasionally, 1,3-1,4-β-glucanase activity was detected by including the substrate lichenan (0.2%) on gels and by using the method of Beguin (1). Basically, SDS was removed by soaking the gels with 25% isopropanol in 50 mM acetate buffer (5 mM β-mercaptoethanol [pH 5.5]) for 30 min and then for an additional 12 h at 4°C with 50 mM acetate buffer (pH 5.5) containing 5 mM β-mercaptoethanol and 1 mM EDTA. Once proteins had been renatured, β-glucanase activity was located by incubating the gel in 50 mM acetate buffer (pH 5.5) at 30°C for 12 h and then staining it by immersion in the Congo red dye solution.

Southern blotting analysis. Transfer of DNA from gels onto nitrocellulose filters and subsequent probing by labeled DNA was done by the method of Southern (34). Radioactive labeling at the 5′ end of DNA fragments was carried out by using [γ-^{32}P]ATP and T4 polynucleotide kinase as described by Maxam and Gilbert (19). Hybridization was done under stringent conditions by incubating at 65°C in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7])–10 mM Tris hydrochloride (pH 7.4)–5× Denhardt solution–0.1% SDS. This was followed by extensive washing at 65°C in 0.2× SSC–0.1% SDS. Alternatively, less stringent conditions were used: incubation at 50°C in 6× SSC–10 mM Tris hydrochloride (pH 7.4)–10× Denhardt solution–0.1% SDS, followed by several washes at the same temperature in 0.2× SSC–0.1% SDS.

Cell fractionation. Fractionation of exocellular, periplasmic, and cytoplasmic/membrane-bound 1,3-1,4-β-D-glucanase from different *E. coli* transformants was carried out by the osmotic shock method of Neu and Heppel (26) as modified by Koshland and Botstein (14). For the same purposes, spheroplasts from irradiated maxicells were obtained by the protocol of Minsky et al. (21). As controls, alkaline phosphatase was assayed as a periplasmic marker enzyme and β-galactosidase was assayed as a cytoplasmic marker enzyme. β-Galactosidase activity and alkaline phosphatase activity were measured as previously described by Miller (20) and Tommassen and Lugtenberg (37), respectively.

Maxicells. For the identification of plasmid-encoded polypeptides, the maxicell method of Sancar et al. (30) as modified by Calhoum and Gray (4), was used. After UV treatment, the cells were incubated at 37°C for 1 h in supplemented M9 minimal medium and then for an additional 16 h under the same conditions but in the presence of D-cycloserine (200 μg/ml). Incorporation of L-[^{35}S]methionine into proteins proceeded for 1 h of incubation in the same system supplemented with the isotope. The cells were then washed with M9 minimal medium, suspended in lysis buffer (50 mM Tris hydrochloride, 1% SDS, 1% β-mercaptoethanol, 5% glycerol [pH 6.8]), boiled for 3 min, and precipitated by the addition of 6 volumes of acetone. After centrifugation, the pellet was suspended in electrophoresis buffer, boiled for 5 min, and loaded onto the gel. Proteins were located by autofluorography of the dried gels with a Kodak XAR-5 film. Pulse-chase experiments on irradiated maxicells were performed as previously described by Koshland and Botstein (15) and by Minsky et al. (21). Basically, irradiated maxicells were subjected to a 30-s exposure to L-[^{35}S]methionine followed by the addition of excess unlabeled methionine and Casamino Acids (chase). At intervals, portions of the culture were immediately transferred to prechilled tubes containing chloramphenicol (50 μg/ml) in ethanol. The cells in each sample were lysed as previously described, and proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE).

RESULTS

Isolation of a gene coding for 1,3-1,4-β-glucanase activity in *B. circulans* WL-12. The experimental approach used for the molecular cloning of the structural gene coding for the 1-3,1-4-β-glucanase of *B. circulans* WL-12 was to select clones of *E. coli* with the ability to hydrolyze 1-3,1-4-β-glucans, after transforming them with a genomic library of *B. circulans*. As has been reported earlier (5, 11) and confirmed in our case with both culture supernatants and intact cells, *E. coli* lacks β-glucanase activity and is unable to hydrolyze lichenan; it is therefore a feasible host for this experimental approach.

A partial *Sau*3A genomic library of *B. circulans* WL-12 DNA, consisting of fragments between 3 and 30 kilobases (kb), was constructed within the *Bam*HI site of plasmid pBR322. The ligation mixture was used to transform *E. coli* DH1; the transformants were selected on LB-ampicillin plates and then replica plated in LB-tetracycline to test for the presence of inserts. About 3,200 Amp^r Tet^s *E. coli* transformants were then screened for 1,3-1,4-β-glucanase

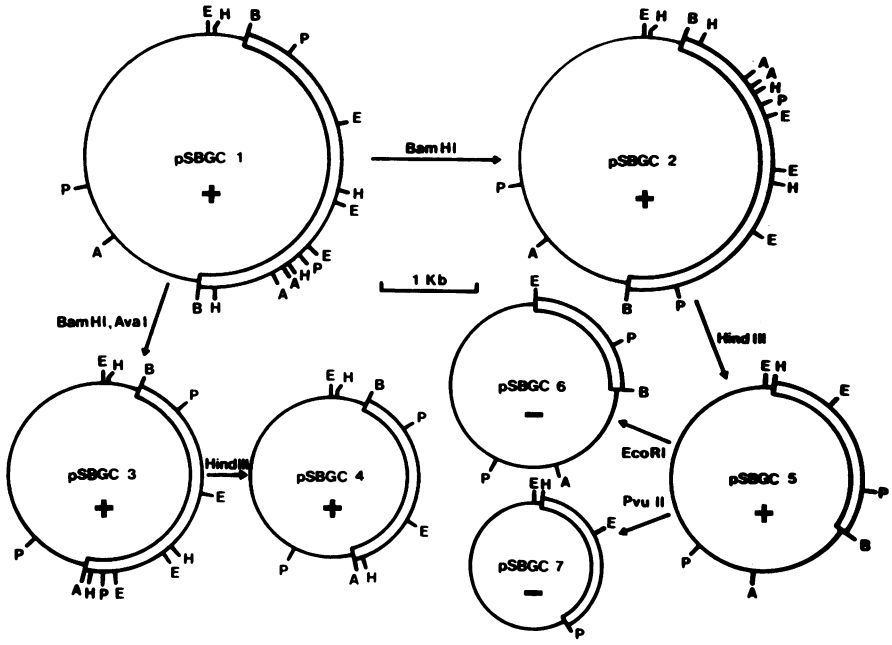

FIG. 1. Boundaries of the *BGC* gene. Restriction endonuclease maps of the *B. circulans* DNA inserts contained in different recombinant plasmids. Restriction endonucleases: A, *Ava*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II. The boxed areas represent the *B. circulans* DNA. Symbols (+ or −) show the production of 1,3-1,4-β-glucanase (*BGC* activity) by *E. coli* strains transformed with the different plasmids.

activity by their ability to form haloes on LB-ampicillin medium supplemented with lichenan. Among them, three showed areas of β-glucan degradation when the plates were assayed by the Congo red method. Restriction digests of minipreparations of the plasmids harbored by these clones revealed that all three contained in the same orientation an identical *Bam*HI-*Bam*HI insert of about 3.8 kb. The recombinant plasmid, named pSBGC1, when used to retransform *E. coli* HB101, conferred the ability to degrade lichenan upon all of the recipient clones.

Restriction endonuclease analysis. Endonuclease digestion of plasmid DNA with a variety of different enzymes resulted in a preliminary physical restriction map of the initial insert contained in pSBGC1. Based on the restriction map, a series of subcloning experiments was performed to delineate the extent of the region coding for β-glucanase production. Plasmid pSBGC2 was obtained by subjecting pSBGC1 DNA to total digestion with *Bam*HI and ligating the 3.8-kb fragment purified from an agarose gel in the opposite orientation into the single *Bam*HI site of pBR322. Plasmid pSBGC3 was constructed by subcloning the 2.9-kb *Bam*HI-*Ava*I pSBGC1 fragment into the *Bam*HI-*Ava*I sites of pBR322. By using different enzymes, several fragments were deleted from these two plasmids, and the plasmids were religated, yielding the following: pSBGC4 (obtained from pSBGC3 by partial digestion with *Hin*dIII), pSBGC5 (pSBGC2 digested with *Hin*dIII), pSBGC6 (pSBGC5 digested with *Eco*RI), and pSBGC7 (pSBGC5 digested with *Pvu*II). The restriction map deduced and the subcloning strategy followed are shown in Fig. 1, together with the glucanolytic activity determined by the resulting subclones on LB-ampicillin-lichenan plates. From these experiments, we concluded that the β-glucanase gene was contained within the 1.9-kb *Bam*HI-*Hin*dIII fragment subcloned in plasmid pSBGC5, since this was the smallest fragment able to encode the ability to degrade lichenan. Thus, the *BGC* gene would extend from the left of the *Hin*dIII site to somewhere between the *Bam*HI and *Pvu*II sites, because the plasmids obtained by digesting this sequence with either *Eco*RI or *Pvu*II failed to confer β-glucanase activity (pSBGC6 and pSBGC7, respectively). In addition, the expression of the gene was not affected by its orientation in the vector, since when the containing fragment was placed in opposite orientations (plasmids pSBGC1 and pSBGC2) both clones expressed β-glucanase activity, suggesting that the cloned fragment carries the complete structural gene together with its own promoter.

Southern blotting confirmed that the fragment cloned in pBR322 was derived from the chromosomal DNA of *B. circulans* WL-12. Total DNA from the strain used to construct the genomic library was totally digested with the restriction endonuclease *Hin*dIII and subjected to Southern analysis with the ^{32}P-labeled 1.4-kb *Bam*HI-*Eco*RI fragment from pSBGC5. Blot hybridization showed a single *Hin*dIII band of about 2.2 kb from the genomic DNA of *B. circulans*

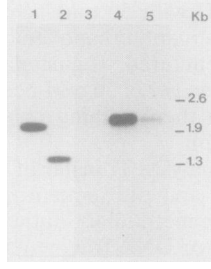

FIG. 2. Hybridization analysis of genomic DNA from *E. coli* HB101 (lane 3), *B. circulans* WL-12 (lane 4), *B. circulans* NRRL-NRS 397 (lane 5), and the recombinant plasmid pSBGC5 (lanes 1 and 2) with the *Bam*HI-*Eco*RI fragment of plasmid pSBGC5 (^{32}P labeled with T4 polynucleotide kinase) as a probe. Samples were digested by *Bam*HI and *Hin*dIII (lane 1), *Bam*HI and *Eco*RI (lane 2), or *Hin*dIII (lanes 3 to 5).

TABLE 1. Substrate specificity of the β-glucanase enzyme produced by *E. coli* transformants[a]

Strain and growth medium	Enzyme activity (mU/10^8 cells)			
	Laminarin	Pustulan	Lichenan	CMC[b]
B. circulans WL-12				
LB	579	476	4,910	233
M9	6,683	6,316	6,341	581
E. coli HB101(pSBGC1)				
LB	1.4	2.6	32.1	3.0
M9	1.1	2.2	35.7	3.5

[a] Supernatants from overnight cultures of different strains grown in LB medium (with 2% glucose as a carbon source) or in M9 minimal medium (with 0.5% mannitol as a carbon source) were concentrated by ultrafiltration and dialyzed against acetate buffer (50 mM, pH 5.5) before the enzymatic assays were performed. *E. coli* HB101 without pSBGC1 did not produce any β-glucanase activity.

[b] CMC, Carboxymethyl cellulose.

WL-12 hybridizing to the cloned gene (Fig. 2). An identical band was present in total DNA from *B. circulans* NRRL-NRS 397 subjected to the same conditions (Fig. 2), although in this case only a slight hybridization was obtained. However, no sequences complementary to the labeled fragment were detected in the *E. coli* DNA digestion. Furthermore, no hybridization was found on genomic DNA from other 1,3-1,4-β-glucanase-producing *Bacillus* strains assayed, such as *B. amyloliquefaciens*, *B. polymyxa*, *B. subtilis*, and *B. coagulans*, even under low-stringency conditions (data not shown).

Expression and characterization of the β-glucanase enzyme produced by *E. coli*. To confirm that the cloned gene did in fact code for the 1,3-1,4-β-glucanase of *B. circulans* WL-12, enzymatic assays were performed in preparations obtained from *E. coli* cells transformed with the isolated recombinant plasmids. Culture supernatants from overnight cultures of HB101(pSBGC1) were screened according to their ability to hydrolyze lichenan (1,3-1,4-β-glucan), laminarin (1,3-β-glucan), pustulan (1,6-β-glucan), and carboxymethyl cellulose (1,4-β-glucan) to assess the substrate specificity of the enzyme. There was no apparent hydrolysis of either laminarin or pustulan, and only a slight degradation of carboxymethyl cellulose was observed (Table 1). However, lichenan was hydrolyzed, indicating that the enzyme produced was specific for mixed-linkage 1,3-1,4-β-glucan. In addition, enzymatic samples obtained from both *B. circulans* WL-12 and from different *E. coli* transformants were used to digest lichenan, and the digestion products were analyzed by paper chromatography; in all the cases, the major end products were di- and oligosaccharides, together with minor amounts of glucose (data not shown), confirming the type of endo-acting activity assigned for this enzyme. From the same data (Table 1), it is of interest to note the different pattern of regulation of this activity compared with those of the other hydrolytic enzymes assayed. Thus, as has been previously reported (7), 1,3-β-glucanase and 1,6-β-glucanase in the parental *B. circulans* WL-12 are subjected to catabolite repression with a significant increase (11- to 13-fold) in the specific activity in the absence of glucose; by contrast, the glucanolytic activity against lichenan appears to be constitutive, with a similar level of synthesis regardless of the carbon sources in the media, either glucose (LB medium), mannitol (M9 medium), or even the substrate lichenan (data not shown). With regard to *E. coli*, the synthesis of 1,3-1,4-β-glucanase also seems to be constitutive, and no effect of the carbon source was observed.

To estimate the molecular size of the enzyme produced by the transformants, electrophoretic analysis of proteins present in concentrated culture supernatants was carried out under denaturing conditions. An extra band of about 40.5 kilodaltons (kDa) was present in culture fluids from *E. coli*(pSBGC1) compared with culture supernatants from nontransformed strains or from strains transformed with the vector plasmid pBR322 (Fig. 3A). By applying the enzymo-

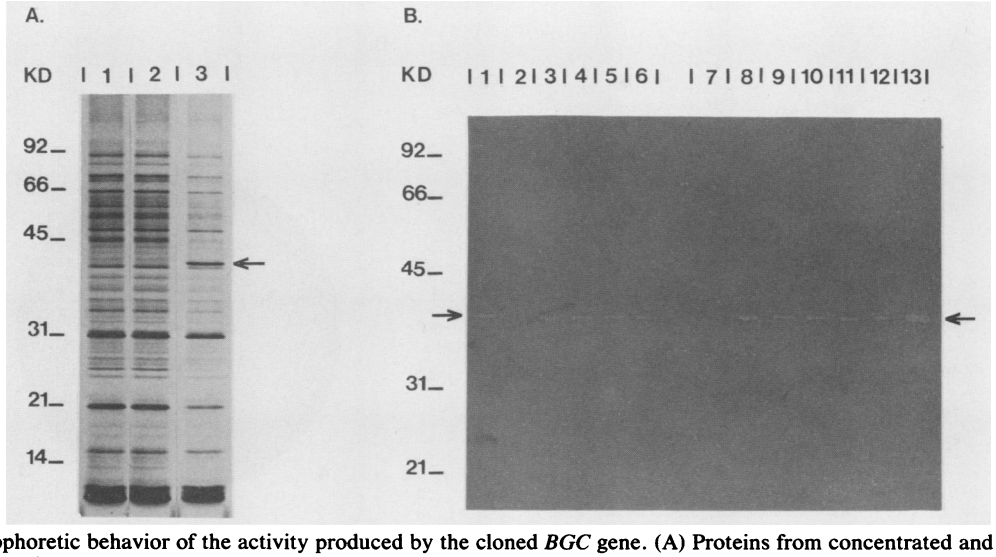

FIG. 3. Electrophoretic behavior of the activity produced by the cloned *BGC* gene. (A) Proteins from concentrated and dialyzed culture supernatants of *E. coli* DH1 strains were separated on 8 to 12% polyacrylamide gradient gels (0.1% SDS) and stained with silver nitrate. Lanes: 1, untransformed *E. coli*; 2, *E. coli* carrying pBR322; 3, *E. coli* carrying pSBGC1. Molecular masses of marker proteins are indicated on the left in kilodaltons (KD). (B) Zymogram of *B. circulans* WL-12 and *E. coli* exocellular proteins separated by SDS-PAGE (10% polyacrylamide, 0.1% SDS) containing the substrate lichenan (0.2%). Clear zones indicated active 1,3-1,4-β-glucanase. Lanes: 1 and 12, *B. circulans* WL-12; 2, *E. coli* DH1(pBR322); 3 to 6, *E. coli* C600, HB101, DH1, and CSR603, respectively, transformed with pSBGC1; 7, untransformed *E. coli* HB101; 8 to 11, *E. coli* HB101 transformed with pSBGC2, pSBGC3, pSBGC4, and pSBGC5, respectively; 13, irradiated *E. coli* CSR603(pSBGC5).

TABLE 2. Location of 1,3-1,4-β-glucanase in different fractions of transformed *E. coli* strains[a]

E. coli strain and fraction	Activity (% of total)		
	β-Glucanase	β-Galactosidase	Alkaline phosphatase
HB101(pSBGC5)			
Exocellular	37	3	2
Periplasmic	62	2	71
Cellular	1	95	27
DH1(pSBGC5)			
Exocellular	37	1	6
Periplasmic	57	2	69
Cellular	6	97	25
C600(pSBGC5)			
Exocellular	31	0	1
Periplasmic	67	3	88
Cellular	2	97	11
CSR603(pSBGC5)			
Exocellular	57	0	32
Periplasmic	43	1	64
Cellular	0	99	4

[a] Exponentially growing cells removed by centrifugation from culture fluids were subjected to fractionation by the osmotic shock procedure. Exocellular refers to the activity present in the culture supernatant, periplasmic refers to activity released by the osmotic shock treatment, and cellular refers to the cytoplasmic membrane-bound activity.

graphic technique of Beguin (1) to detect glucanase activity of renatured proteins separated by SDS-PAGE (Fig. 3B), it became clear that the ability to degrade lichenan was determined by a single polypeptide of similar size, present in both culture fluids from the parental strain *B. circulans* WL-12 and in supernatants from cultures of transformed *E. coli* (containing pSBGC1, pSBGC2, pSBGC3, pSBGC4, or pSBGC5). *E. coli* containing a negative control plasmid expressed no detectable activities.

Fate of cloned β-glucanase in *E. coli*. The presence of hydrolysis haloes on plates with lichenan showed that at least a fraction of the glucanase produced by the strains of *E. coli* transformed with recombinant plasmids containing the β-glucanase gene was extracellular, either as a result of secretion of the protein or as a result of partial lysis of the colonies. To determine the fate of the β-glucanase expressed in the transformants, studies were conducted on subcellular location, separating different cellular fractions by osmotic shock (extracellular, periplasmic, and cytoplasmic/membrane bound) and evaluating in them the capacity to release reducing groups from lichenan. The efficiency of the cell fractionation procedure was confirmed by assaying the catalytic activities of alkaline phosphatase and β-galactosidase in each of the different fractions. There was little difference in the relative location of β-glucanase activity for *E. coli* HB101, DH1, and C600; on the average, 62% was secreted into the periplasmic space, 35% being detected extracellularly and 3% remaining bound to the cellular fraction (Table 2). In all cases, the controls of alkaline phosphatase and β-galactosidase were those expected for the cellular location of these enzymatic markers. In the case of *E. coli* CSR603, the strain later used in maxicell experiments, the subcellular distribution observed was appreciably different from that obtained for the aforementioned strains, with a 57% release into the culture medium of the β-glucanase activity detected and 43% remaining in the periplasmic space. This finding, in contrast with those obtained in the other strains assayed, can be understood on evaluating the periplasmic marker alkaline phosphatase, which is likewise released into the extracellular space at a higher proportion compared with those in the other strains employed.

Expression of the β-glucanase gene in *E. coli* maxicells. To gain further information on the synthesis and expression in *E. coli* of the cloned β-glucanase, maxicell experiments were carried out in *E. coli* transformants. The maxicell strain CSR603 containing pSBGC1, pSBGC2, pSBGC4, pSBGC5, or the vector pBR322 was UV irradiated, and the polypep-

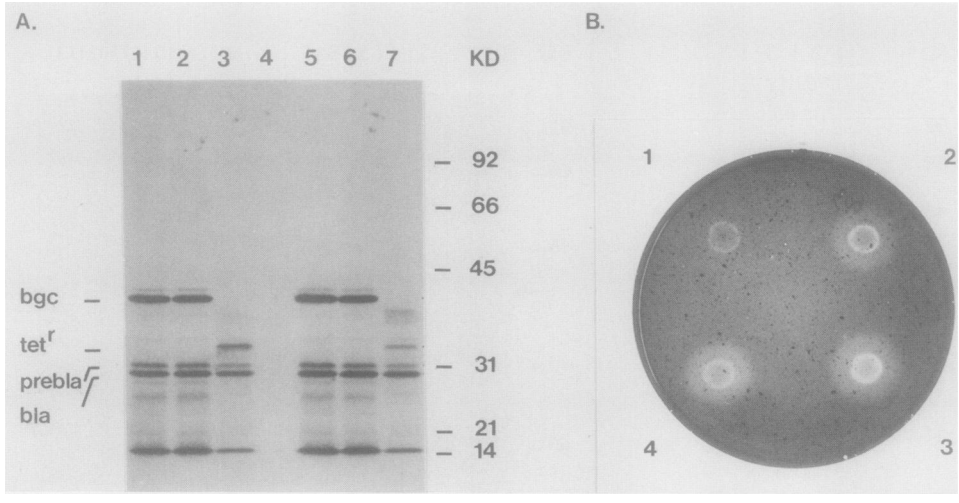

FIG. 4. Analysis of plasmid-encoded proteins synthesized in UV-irradiated maxicells. (A) Autoradiogram of labeled polypeptides, separated by SDS-PAGE (10% polyacrylamide, 0.1% SDS), produced in *E. coli* CSR603 (lane 4) and in *E. coli* CSR603 containing pBR322 (lanes 3 and 7), pSBGC1 (lane 1), pSBGC2 (lane 2), pSBGC4 (lane 5), or pSBGC5 (lane 6). prebla and bla indicate the positions of pre-β-lactamase and mature enzyme, respectively; bgc indicates the position of 1,3-1,4-β-glucanase. (B) β-Glucanase assay in irradiated maxicells. Before radioactive uptake, samples of irradiated maxicells were transferred to LB-ampicillin-lichenan plates. After incubation for 12 h at 37°C, β-glucanase activity was detected by staining with Congo red indicator: *E. coli* CSR603 carrying pBR322 (sample 1), pSBGC1 (sample 2), pSBGC2 (sample 3), or pSBGC5 (sample 4). No growth was observed in any case.

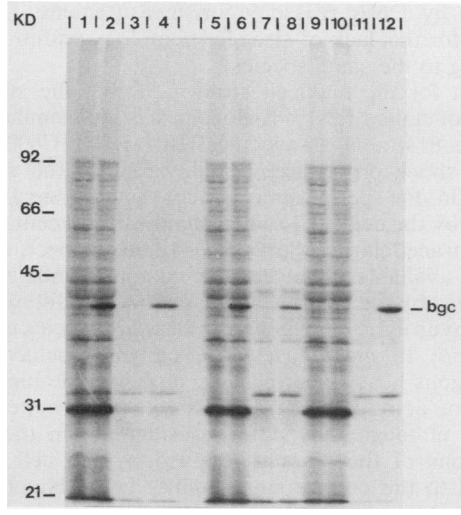

FIG. 5. Fractionation analysis of labeled maxicells transformed with pBR322 and pSBGC5. Two cultures of *E. coli* CSR603 transformed with pBR322 and pSBGC5 were irradiated. Each culture was distributed in three samples, and plasmid-encoded proteins were labeled in the presence of L-[^{35}S]methionine. After labeling, one of the samples of each culture was used to determine the proteins present in culture supernatants and in total cell extracts. Cells from the other two samples were fractionated into periplasmic and cytoplasmic/membrane-bound proteins by the osmotic shock method and by spheroplasting. Electrophoresis was carried out on a 10% polyacrylamide–0.1% SDS gel with whole-cell extracts of *E. coli* CSR603 carrying pBR322 (lane 1) and pSBGC5 (lane 2) and corresponding culture supernatants of cells carrying pBR322 (lane 3) and pSBGC5 (lane 4). Fractionation by osmotic shock treatment: cytoplasmic/membrane-bound fraction of cells carrying pBR322 (lane 5) and pSBGC5 (lane 6) and corresponding periplasmic fraction released (pBR322, lane 7; pSBGC5, lane 8). Fractionation by conversion of cells to spheroplasts: cytoplasmic/membrane-bound fraction of cells carrying pBR322 (lane 9) and pSBGC5 (lane 10), and corresponding periplasmic fraction released (pBR322, lane 11; pSBGC5, lane 12).

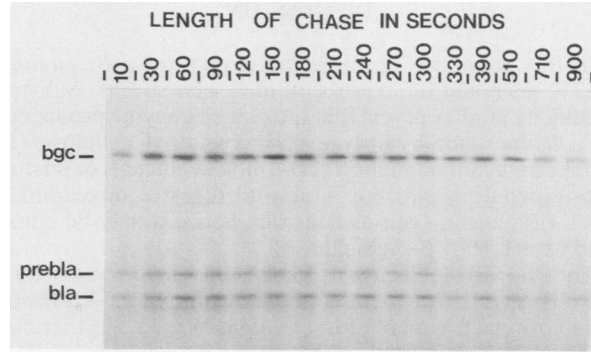

FIG. 6. Autoradiogram of labeled proteins, separated by SDS-PAGE (10% polyacrylamide, 0.1% SDS), from pulse-chase analysis in irradiated maxicells. The number above each slot indicates the time (in seconds) after the addition of the chase. prebla and bla indicate the positions of pre-β-lactamase and mature enzyme, respectively; bgc indicates the position of 1,3-1,4-β-glucanase.

tides encoded by the plasmids were analyzed by SDS-PAGE after uptake of L-[^{35}S]methionine. The strain harboring the vector pBR322 alone gave rise to three bands of M_r 35,000, 31,000, and 29,000 (Fig. 4), the highest one corresponding to the Tet^r gene product and the latter two corresponding to the Amp^r gene products (pre-β-lactamase and mature β-lactamase, respectively). Cells containing recombinant plasmids lack the Tet^r gene product but produce pre-β-lactamase and mature proteins together with a major additional band, encoded by the insert, with a molecular mass of about 40.5 kDa, similar in size to that estimated for the protein with β-glucanase activity detected in culture supernatants of strains transformed with the same recombinant plasmids (Fig. 3). In each case, production of an active β-glucanase in the maxicell strain *E. coli* CSR603 was monitored on lichenan plates by the Congo red assay (Fig. 4B).

Location of the product of the cloned gene was also assayed in irradiated maxicells after uptake of the labeled amino acid. After culture supernatant separation, fractionation of the total cell extract activity in periplasmic and cytoplasmic/membrane-bound 1,3-1,4-β-glucanase was carried out in parallel samples by the osmotic shock method and by spheroplasting. SDS-PAGE of the different fractions is shown in Fig. 5. Lanes 2 and 4 show the extracellular fate of part of the β-glucanase synthesized. In culture supernatants (lane 4) a 40.5-kDa protein appeared, representing approximately 50% of the amount detected in total cell extracts (lane 2). Fractionation of this cell-associated activity varied considerably depending on the method used. Thus, although in the sample treated by osmotic shock partial release of the 40.5-kDa protein occurred (periplasmic fraction, lane 8), with a large part remaining in the cytoplasmic/membrane fraction (lane 6), the generation of spheroplasts led to total release of the enzyme (lanes 10 and 12), which suggests a predominantly periplasmic location.

Pulse-chase experiments. In an attempt to gain preliminary evidence concerning the type of processing of the product of secretion coded by the cloned gene, pulse-chase experiments were carried out under the conditions reported by Minsky et al. (21) and by Koshland and Botstein (15) to study the kinetics of synthesis of the TEM β-lactamase. Figure 6 presents the results from a pulse-chase experiment in which a 30-s exposure to L-[^{35}S]methionine was followed by incubation with excess unlabeled amino acid. As expected, two bands corresponding to the pre-β-lactamase (31.5 kDa) and mature β-lactamase (29 kDa) appeared in the first samples taken after the addition of the cold amino acid; as the chase proceeded there was a progressive disappearance of the precursor form, in agreement with previous reports suggesting that β-lactamase is processed posttranslationally. However, in contrast to β-lactamase, in the case of β-glucanase only one band migrating at the same position as the mature enzyme could already be detected 10 s after the addition of the unlabeled amino acid, and no radioactivity was found in higher-molecular-weight products corresponding to a putative precursor.

A similar case has recently been reported by van Dijl et al. (38) for the processing of *Bacillus licheniformis* α-amylase in *E. coli*. As these authors suggest, two possible explanations can be envisaged for the observation that only mature product is detected. The first is that the processing of the preprotein is too fast to be detectable under the experimental conditions used; the alternative hypothesis is that the protein can be cotranslationally translocated and processed. Although at present we cannot decide which possibility is correct, the fact that only one form is detected seems to indicate, as in the case of α-amylase, that the rate of processing of β-glucanase is very high and that its synthesis may be coupled with the processing of the precursor preprotein. Additional experiments should address this aspect after a structural characterization of the cloned gene.

DISCUSSION

In this paper we describe the cloning of a *B. circulans* WL-12 gene and its expression in *E. coli* strains. Substrate specificity studies reveal that the cloned enzyme produced in *E. coli* does not hydrolyze carboxymethyl cellulose (1,4-β-linked glucan), laminarin (1,3-β-linked glucan), or pustulan (1,6-β-linked glucan) but is able to digest a mixed-linkage 1,3-1,4-β-glucan, such as lichenan; hence it may be considered as a 1,3-1,4-β-D-glucanase.

The cloned β-glucanase gene is located on a 1.9-kb *Bam*HI-*Hin*dIII fragment contained within a 2.2-kb *Hin*dIII chromosomal fragment of *B. circulans* WL-12. Furthermore, the evidence presented in this paper is consistent with the idea that the cloned gene remains associated with its own promoter and is able to direct its own transcription and translation in *E. coli*. On one hand, subcloning experiments show that enzyme production is independent of the orientation of the fragment with respect to plasmid promoters. On the other, maxicell experiments show that when present in opposite orientations the β-glucanase-containing fragment produces the encoded polypeptide in equivalent amounts, indicating that transcription of the gene arises from the normal gene promoter and not from a vector promoter via readthrough transcription.

The β-glucanase enzyme produced and secreted in *E. coli* apparently has the same size as that found in the parental *Bacillus* strain, as shown by electrophoretic analyses of culture supernatants of transformed cells and of the polypeptides synthesized in irradiated maxicells. Although differences in size due to the limitations in molecular weight determination by SDS-PAGE cannot be ruled out, the fact that the products synthesized in both organisms are very similar points to a correct translation and processing of the protein in the heterologous host. It should be emphasized that only one additional major band was present in maxicell extracts, indicating that the β-glucanase remained intact and was not degraded by the proteases of *E. coli*. In any case, it should be noted that there is a considerable discrepancy with respect to other similar enzymes regarding the mode of action when they have been previously cloned and sequenced from other *Bacillus* species. Thus, Murphy et al. (23) and Hofemeister et al. (12) reported the nucleotide sequence of two 1,3-1,4-β-glucanase genes from *B. subtilis* and *B. amyloliquefaciens*, calculating molecular masses for the pre-β-glucanases of 26,950 and 27,288 Da, respectively; except for the putative signal peptide sequence, the translated regions show an extensive homology of about 90%, confirming the proposed close proximity of these two taxa (28). Taking into account that we estimated the molecular mass of the mature product at 40.5 kDa and that the putative precursors of the aforementioned enzymes are about 3 kDa larger than the secreted forms, it may be concluded that the 1,3-1,4-β-glucanase from *B. circulans* WL-12 is about twice the size of the β-glucanases from the other *Bacillus* species. This remarkable difference is corroborated by the apparent lack of homology as shown by Southern analysis, where no hybridization was detected between the cloned gene and genomic sequences from the other *Bacillus* species, even under conditions favoring hybridization in the absence of strong homology. In this sense, it is appropriate to remember that the only sequences hybridizing to the cloned gene were found in a similar-sized restriction fragment from a different strain of *B. circulans*, NRRL-NRS 397, although only a slight hybridization was obtained; the wide heterogeneity attributed to the organisms included in this particular taxon, revealed by DNA reassociation measurements (24), may account for this lack of stronger homology within members belonging to the same species.

Except for the maxicell strain CSR603, the subcellular location of cloned 1,3-1,4-β-glucanase is very similar in all of the *E. coli* strains assayed (HB101, DH1, C600) by the osmotic shock procedure. Roughly 62% of the activity is present in the periplasmic space, with a small portion retained by the cells (3%) and a significant percentage found in the extracellular fraction (35%). In this respect, extensive data are available about cellular compartmentalization, as determined by the osmotic shock method, of different extracellular hydrolytic enzymes from *Bacillus* species expressed in *E. coli*. In most cases, the enzymes behave in the heterologous host as periplasmic proteins, the highest proportion of activity being associated with the periplasmic fraction, although they differ considerably in the relative proportions of the amount retained by the cell and that liberated to the culture supernatant. Thus, Hinchliffe (11) determined that the 1,3-1,4-β-glucanase of *B. subtilis* NCIB 8565 expressed in *E. coli* resides mainly in the periplasmic space (57%), with 26% of the activity released into the culture fluid and 17% retained by the cells. In a similar way, Cornelis et al. (6) found that a cloned *B. coagulans* α-amylase was also primarily located in the periplasm (83%), although in this case the intracellular fraction was higher (13%) than that released into the extracellular space (4%). A similar phenomenon was observed by Sashihara et al. (32) for a 1,4-β-glucanase gene from an alkalophilic *Bacillus* strain. By contrast, as described by Robson and Chambliss (29), the 1,4-β-glucanase of *B. subtilis* DLG when expressed in *E. coli* is mostly retained within the cytoplasm of the transformants (63%), with a portion associated to the periplasmic fraction (37%) and only a slight proportion (<1%) secreted to the surrounding medium. Our results are in fairly close agreement with most of the aforementioned data, and the fact that the activity present in the extracellular and periplasmic fractions accounts for 97% of the total suggests that the heterologous host can correctly process the enzyme for secretion and that the signal sequence of the protein is efficient in promoting its transfer through the *E. coli* inner membrane.

However, note should be taken of the discrepancy in the results concerning the localization of the product coded by the gene cloned in the maxicell strain CSR603. Thus, although when the subcellular distribution was determined by assaying the enzyme activity present in the different fractions almost all of this activity was found to be located outside the cytoplasm, when the distribution of the labeled polypeptide was determined in irradiated maxicells a significant part of the enzyme remained associated with the intracellular fraction. We are unaware of the reasons for such a discrepancy, although it could be due to a different sensitivity to the osmotic shock brought about by the differing physiological states of the cells in both assays. In this sense, Oudega and Mooi (27) have proposed that growing cells are more susceptible to this method than stationary cells and that irradiated maxicells can be expected to behave in localization studies like stationary *E. coli* cells. Confirmation of this is that when subcellular fractionation was performed by spheroplasting, practically all of the encoded polypeptide was released.

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