APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1988, p. 302–308
0099-2240/88/020302-07$02.00/0

Vol. 54, No. 2

Characterization of an Endoglucanase from *Pseudomonas fluorescens* subsp. *cellulosa* Produced in *Escherichia coli* and Regulation of the Expression of Its Cloned Gene

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Received 16 October 1987/Accepted 5 November 1987

Several enzymatic properties of an endoglucanase produced in *Escherichia coli* by a gene from *Pseudomonas fluorescens* subsp. *cellulosa* were investigated. Gel filtration revealed a single peak of M_r 36,000 with endoglucanase activity. The pH optimum of the enzyme was 7.0. Carboxymethyl cellulose and barley β-glucan (mixed β-1,3 and 1,4 linkages) were good substrates, but not laminarin (β-1,3 linkages), amylose, filter paper, microcrystalline cellulose (Avicel), or cellotriose. The mode of action was typical of an "endo"-acting enzyme. Taken together, these properties do not correspond to those of any of the endoglucanases described in *P. fluorescens* subsp. *cellulosa*. Consequently, the gene was designated *eglX*. The enzyme was sensitive to end-product inhibition by cellobiose but was only moderately inhibited by glucose. The enzyme was formed constitutively in *E. coli* throughout the growth phase. Urea had no effect on endoglucanase synthesis, but glucose acted as a catabolite repressor. The formation of the enzyme in *E. coli* was partially dependent on cyclic AMP.

The group of enzymes that perform the degradation of cellulose are known collectively as cellulases. They include hydrolytic enzymes which fall into one of two groups according to the site of cleavage in the polysaccharide chain. Cleavage at a specific site adjacent to the nonreducing end of the polysaccharide chain is catalyzed by exoenzymes, of which cellobiohydrolases (EC 3.2.1.91) are the most often described. Hydrolysis at random sites is catalyzed by endoglucanases (EC 3.2.1.4). In cellulolytic microorganisms, they often occur in multiple forms having different molecular weights, carbohydrate contents, and modes of action (10). Large-scale enzymatic hydrolysis of cellulose has also been envisioned as a route towards conversion of cellulose to fuels, food, and chemicals. However, cellulolysis is a relatively slow process compared with other enzymatic reactions, and the conversion of cellulose to glucose via cellulases has been hampered by the low yields and high cost of the microbial enzymes used (8).

The macromolecular and insoluble nature of cellulose implies that a coordinate regulation of the various enzymes of the cellulase complex must occur without direct interaction between the substrate and intracellular effector molecules (1). These regulation mechanisms are poorly understood, although an understanding of them is essential if cellulase yields are to be improved to the point of economic feasability in bioconversion processes. The synthesis and activity of cellulase enzymes are affected by induction, catabolite repression, and end-product inhibition (17). Catabolite repression is defined as the mechanism by which organisms decrease the rate of synthesis of catabolic enzymes when useful catabolites accumulate in the cell. This mechanism is mediated by the cyclic AMP (cAMP) system in *Escherichia coli*, in which it has been extensively studied (2). Many microorganisms require the presence of inducers to actively produce cellulases. In this respect, glucose, cellobiose, sophorose, and other soluble sugars can act as repressors or inducers, depending on the organism (9).

A genetic approach to cellulose hydrolysis will make possible a closer study of some aspects of the expression and structure of cellulase genes and of the corresponding enzymes and may lead to enhanced cellulase production. Our specific emphasis has been on the cellulolytic soil microorganism *Pseudomonas fluorescens* subsp. *cellulosa* (NCIB 10462) (29), because its cellulase complex has been studied in some detail. It comprises extracellular and cell-bound cellulases that are glycosylated and display distinct substrate specificities (37–40). Intracellular and extracellular cellulases show different regulation patterns (36). Extracellular cellulase formation is constitutive and is repressed by glucose and glucose disaccharides readily hydrolyzed by the cells (26). The cloning of two endoglucanase genes of *P. fluorescens* subsp. *cellulosa* into *E. coli* (14, 33) is a first step to gaining insight into the factors influencing cellulase production and activity. In this study, we describe some properties of the cellulase encoded by the gene we cloned and compare them with the known characteristics of the cellulases of *P. fluorescens* subsp. *cellulosa* in an attempt to identify the enzyme. We also investigate the regulation of expression of this gene in *E. coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* CA8306 (F⁻ *thi* Δ*cya854*) (5) was used in this study. The deletion of the gene for adenylate cyclase prevents the synthesis of cAMP. *E. coli* AKN2001 was constructed by transducing the wild-type *cya* gene from *E. coli* CL1047 (*met ara pro relA rpsL* HfrC; C. Colson) to CA8306 with phage P1 *vir* (16) and selecting colonies able to grow on maltose as the sole carbon source. Plasmid pRUCL150 is a derivative of pBR322 in which an 11.1-kilobase DNA fragment from *P. fluorescens* subsp. *cellulosa* NCIB 10462 has been cloned at the *Eco*RI site. It encodes resistance to tetracycline and ampicillin and carries a functional cellulase gene (14).

Culture media and growth conditions. Bacteria were routinely grown in L broth containing 1% tryptone (Difco), 1% yeast extract (Difco), and 0.5% NaCl and in a minimal

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medium composed of mineral salts (6), 1 μg of thiamine per ml, 20 μg of tryptophan per ml, 0.1% Casamino Acids (Difco), and 0.4% glycerol. When appropriate, tetracycline (10 μg/ml; Boehringer, Mannheim, Federal Republic of Germany) was added to the medium. β-Galactosidase was induced with isopropyl-β-D-thiogalactoside (IPTG; Boehringer) at a final concentration of 1 mM. For studies on the effect of various compounds on endoglucanase synthesis, *E. coli* cells were grown in minimal medium, and the compound to be tested was added to half of the culture in the early exponential phase; the other half served as a control. Liquid cultures were grown with aeration at 37°C. Growth was monitored by absorbance measurements at 650 nm.

Preparation of samples for enzyme assays. Highly active endoglucanase preparations were obtained from stationary-phase cultures by an osmotic shock in the cold (19). The periplasmic fractions containing the endoglucanase were extensively dialyzed against the citrate-phosphate buffer of McIlvaine (50 mM, pH 7.0) (15) before assays. When necessary, solutions were concentrated in a dialysis bag covered with polyethylene glycol 20,000. For studies on the endoglucanase produced under different conditions, 2.5-ml samples were periodically removed from *E. coli* cultures, and the cells were suspended in 1.5 ml of McIlvaine buffer (50 mM, pH 7.0), sonically disrupted (Braun Sonic Power Co. sonifier, model B12, with microtip, 70 W) four times for 10 s each in the cold, and centrifuged at 13,000 × *g* for 5 min in a benchtop minifuge. The supernatant was used as the enzyme source.

Enzyme assays. β-Galactosidase was assayed by the method of Miller (16). One unit of enzyme activity corresponds to the liberation of 1 μmol of *o*-nitrophenol per min ($\varepsilon = 0.0045\ \mu M^{-1}$). Carboxymethyl cellulose (CMC; type 7LD; Hercules, Rueil Malmaison, France), barley β-glucan and laminarin (Sigma, Deisenhofen, Federal Republic of Germany), amylose (Merck, Darmstadt, Federal Republic of Germany), filter paper (no. 1; Whatman, Maidstone, England), Avicel (microcrystalline cellulose, type SF; Serva, Heidelberg, Federal Republic of Germany), and sugar beet pulp (Raffinerie Tirlemontoise, Tienen, Belgium) were tested as substrates for the endoglucanase. Enzyme was incubated with 0.5% (wt/vol) solutions or suspensions of the substrates in 50 mM McIlvaine buffer, pH 7.0, at 30°C for 30 min. The reducing sugars formed were assayed by the Somogyi-Nelson method (18). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugar (estimated as glucose) per min. Cellotriose was also used as a possible substrate for the endoglucanase. The oligosaccharide (3.5 mg in 50 mM McIlvaine buffer, pH 7.0) was incubated with the enzyme in a total volume of 1 ml at 30°C for various periods of time. The reaction was terminated by heating at 70°C for 10 min. Glucose was determined in the samples with a kit for glucose detection by the glucose oxidase method (Hoffmann-La Roche, Basel, Switzerland) as recommended by the manufacturer. The products were also analyzed by thin-layer chromatography on cellulose. Portions were spotted on sheets of Polygram cell 300 (Macherey-Nagel, Düren, Federal Republic of Germany). A mixture of glucose, cellobiose, and cellotriose was used as the standard. The chromatograms were developed by two consecutive ascending migrations with *n*-butanol–pyridine–water (6:4:3, vol/vol/vol). The chromatograms were revealed with the silver nitrate-acetone reagent (32). Endoglucanase activity was also determined by a viscometric assay. Reaction mixtures consisting of enzyme and 0.75% CMC (type 7MF; Hercules) in 50 mM McIlvaine buffer, pH 7.0, were incubated at 30°C, and the reaction was terminated by heating at 70°C for 10 min. The samples were thermostated at 20°C, and the viscosity was determined with an Ostwald-type viscometer (7). The reciprocal of the specific viscosity (fluidity, $1/n_{sp}$) was calculated by the formula $1/n_{sp} = t_0/t - t_0)$, where t_0 is the efflux time of buffer (17 s) and t is the efflux time of the CMC solution. Enzymatic activity was proportional to the increase in fluidity (23). Proteins were determined by the dye-binding assay (4) with bovine serum albumin as the standard.

Determination of the molecular weight of the endoglucanase by gel filtration. A column (3.0 by 90 cm) was packed with Ultrogel AcA54 (LKB, Broma, Sweden) and equilibrated with 50 mM McIlvaine buffer, pH 7.0. *E. coli*(pRUCL150) periplasmic fractions were concentrated in a dialysis bag to approximately 1 mg of protein per ml (endoglucanase specific activity, 700 mU/mg of protein). The samples loaded on the column contained 2 ml of protein, 1 ml of dinitrophenyllysine (1 mg/ml in McIlvaine buffer), 2 ml of 50% glycerol, and 2 ml of McIlvaine buffer. Protein separation was conducted at 4°C at a flow rate of 40 ml/h, and 8-ml fractions were collected. Proteins eluted from the column were monitored by UV light at 280 nm. Each fraction was tested for endoglucanase activity by spotting 20-μl samples on plates containing 0.1% CMC (type 7LD) in 50 mM McIlvaine buffer, pH 7.0, and 0.8% agar. The plates were incubated overnight at 37°C, and active fractions were detected by the Congo red procedure (27). Molecular weight marker proteins were filtered separately. The elution constant (K_d) of a protein was calculated as described by Freifelder (11).

RESULTS

Molecular weight of the endoglucanase. To determine the molecular weight of the native endoglucanase produced by *E. coli*(pRUCL150), a concentrated periplasmic fraction was applied on a column for gel filtration. A total of 140 fractions were collected and tested for endoglucanase activity by the Congo red procedure. Forty active fractions were detected and further assayed by reducing sugar determination. A single peak corresponding to a K_d of 0.38 was obtained. The deduced molecular weight of the enzyme was 36,000 (Fig. 1).

Mode of action. The hydrolysis of CMC results in a reduction of viscosity (or an increase in fluidity) of the solution and an increase in reducing sugar residues. Viscosity is in direct relation to the length of the polymer molecules, while a reducing group is created by any hydrolytic event regardless of the position of the cleavage site in the polymer. Endoglucanases will therefore cause a sharp decrease in viscosity with a slow liberation of reducing sugars. A mixture of appropriately diluted enzyme and CMC (type 7MF, 0.75% final concentration) in McIlvaine buffer was incubated at 30°C and sampled periodically. The specific viscosity and reducing sugar content of each sample were measured. The curves in Fig. 2 show a typical pattern for an enzyme that catalyzes the internal cleavage of the substrate. This confirmed the endoglucanase nature of the enzyme encoded by the cloned gene.

Substrate specificity. An *E. coli*(pRUCL150) periplasmic extract was examined for its ability to hydrolyze various carbohydrate polymers. The enzyme had specific activities of 740 and 370 mU/mg of protein when CMC and barley β-glucan, containing mixed β-1,3 and β-1,4 linkages, were used as substrates, respectively. The presence of β-1,4 linkages seemed to be required for enzyme activity, since

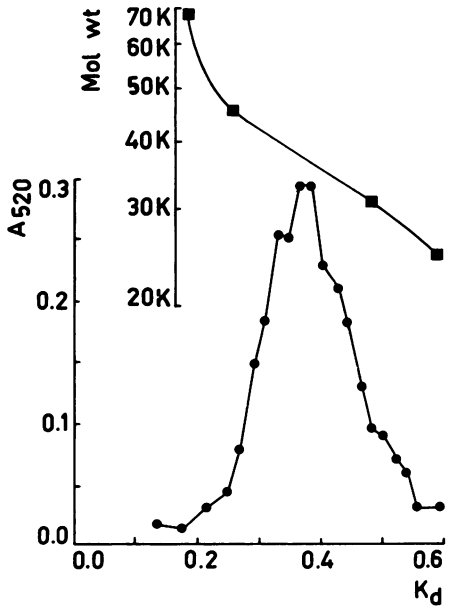

FIG. 1. Molecular weight of the endoglucanase determined by gel filtration. Symbols: ●, absorbance at 520 nm in the reducing sugar assay for endoglucanase; ■, reference curve for M_r determination. Molecular weight markers are bovine serum albumin (68,000), ovalbumin (45,000), DNase I (31,000), and chymotrypsinogen (25,000).

laminarin (β-1,3 linkages) and amylose (α-1,4 linkages) were not degraded. In addition, no activity was displayed towards insoluble cellulosic substrates (filter paper, Avicel, and sugar beet pulp). Extracts of *E. coli*(pBR322) showed no detectable activity towards any of the above substrates.

The possible hydrolytic activity of the endoglucanase towards cellotriose was also investigated. The enzyme was incubated with cellotriose, and samples were removed after 10, 20, and 30 min and 1, 3, and 6 h of reaction. The reaction products were analyzed by two methods. First, glucose was measured by the glucose oxidase test, and second, the reaction products were separated by thin-layer chromatography. The absence of glucose and cellobiose was evidenced by these two analytical procedures. This clearly established that cellotriose was not a substrate for the endoglucanase.

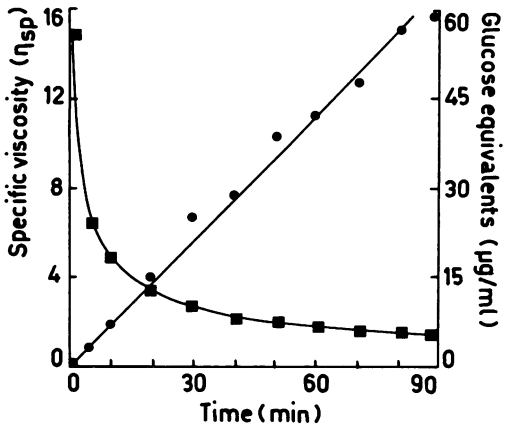

FIG. 2. Decrease in specific viscosity and generation of reducing groups during CMC hydrolysis. Symbols: ■, specific viscosity; ●, reducing power.

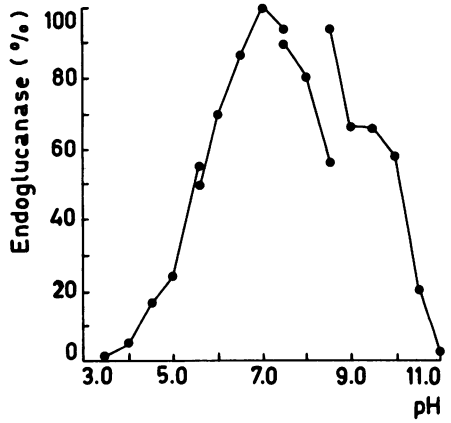

FIG. 3. Influence of pH on enzyme activity. Sodium citrate buffer (50 mM) was used for pH 3.5 to 5.6; sodium phosphate (50 mM) for pH 5.6 to 7.5; Tris hydrochloride (50 mM) for pH 7.5 to 8.5; and glycine-NaOH (50 mM) for pH 8.5 to 11.0.

Physiological properties of the endoglucanase. To study the effect of pH on endoglucanase activity, the enzyme was incubated with CMC in various buffers ranging in pH from 3.5 to 11.0, and the reducing sugars were measured. The curve in Fig. 3 shows a pH optimum of 7.0, with the enzyme retaining 50% of its activity between pH 5.5 and 10.0. The inhibition by glucose and cellobiose of the endoglucanase activity was assayed viscometrically. While the endoglucanase activity was only moderately inhibited by glucose, strong inhibition by cellobiose was observed, with as little as 5 mM cellobiose causing a 50% drop in activity (Fig. 4).

Time course of endoglucanase production in *E. coli* (pRUCL150). *E. coli* AKN2001 harboring plasmid pRUCL150 was grown in L broth and in glycerol minimal medium. Endoglucanase synthesis started at the onset of the exponential phase of growth. Enzyme activity reached a plateau when the culture entered the stationary phase in complete medium, whereas it showed a maximum followed by a decrease in activity in minimal medium. The highest endoglucanase specific activity (approximately 70 mU/mg of protein) was similar in both media, indicating that the relative rate of endoglucanase synthesis was not affected by

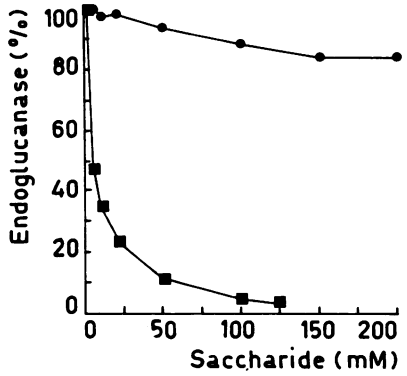

FIG. 4. Effect of glucose and cellobiose on endoglucanase activity. Endoglucanase was added to a mixture containing, in McIlvaine buffer, 0.5% CMC and increasing amounts of glucose (●) or cellobiose (■). The reaction was allowed to proceed for 30 min at 30°C. The reaction was terminated by heating at 70°C for 10 min, and enzyme activity was measured by the viscometric assay.

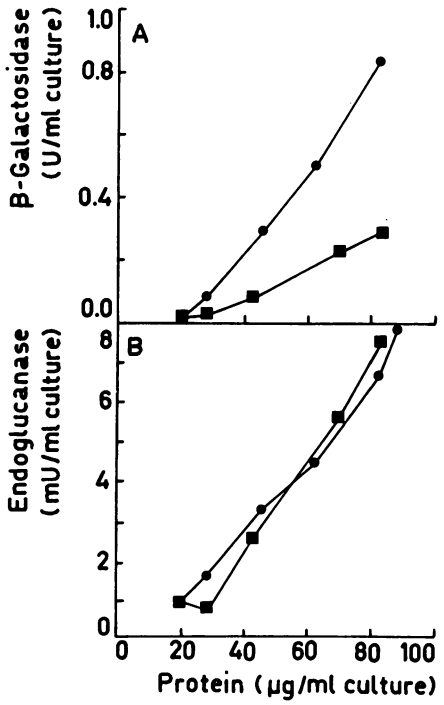

FIG. 5. Effect of 0.6 M urea on enzyme synthesis in *E. coli* AKN2001(pRUCL150) grown in glycerol minimal medium. A culture in early exponential phase (protein concentration, 20 μg/ml of culture) was supplemented with IPTG, and urea was added concomitantly to half of the culture. (A) β-Galactosidase; (B) endoglucanase. Symbols: ●, control culture without urea; ■, culture with urea.

the culture medium used under the present conditions. Of note is that endoglucanase synthesis occurred in the absence of any potential inducer. From these data, it appears that the endoglucanase is produced constitutively in *E. coli*.

Regulation of expression of the endoglucanase gene in *E. coli*. To further investigate the regulation of expression of the endoglucanase gene in *E. coli*, the effects of several compounds added to a minimal culture medium with glycerol as the carbon source were investigated. The substances to be tested were added to early exponentially growing cultures. Samples were removed at various times, and crude cell extracts were assayed for endoglucanase activity.

Since it is known that low concentrations of urea specifically repress the expression of catabolite-sensitive genes in *E. coli*, we examined the effect of the addition of 0.6 M urea on the endoglucanase production by a culture of *E. coli* AKN2001(pRUCL150) (Fig. 5). As a control, β-galactosidase was measured in both the absence and presence of urea (Fig. 5). As expected, β-galactosidase was clearly repressed in the presence of urea. In contrast, similar rates of endoglucanase synthesis were observed under both culture conditions. When glucose was added to a final concentration of 0.2%, repression on both IPTG-induced β-galactosidase and endoglucanase was evident (Fig. 6). When 0.4% glucose was added, less than 20% of the endoglucanase activity of the control culture was measured. Inclusion of CMC in a final concentration of 0.1% had no effect on endoglucanase synthesis (data not shown). In a final experiment, the effect of cAMP was studied with the use of *E. coli* CA8306(pRUCL150). This strain is unable to synthesize cAMP due to a deletion in its adenylate cyclase gene but is otherwise isogenic to AKN2001(pRUCL150). An early-exponential-phase culture was induced for β-galactosidase, and half of the culture was supplemented with 5 mM cAMP; the other half served as a control without cAMP. Addition of the cyclic nucleotide markedly enhanced endoglucanase as well as β-galactosidase synthesis (Fig. 7).

DISCUSSION

The cellulase complex of *P. fluorescens* subsp. *cellulosa* comprises five extracellular cellulases [I-(1) and II to V] and a membrane-bound cellulase C. All are glycoproteins (37–40). The end product of the extracellular cellulases is mainly cellotriose. The membrane-bound cellulase C, but not the extracellular cellulases, is active towards this latter substrate (37). It is therefore assumed that most of the sugar taken up by *P. fluorescens* subsp. *cellulosa* is cellotriose, which is cleaved into glucose and cellobiose by the membrane-bound cellulase C. Cellobiose is further hydrolyzed by an intracellular cellobiase (12). Sequential conversion of cellulase I-(1) to V has been demonstrated to occur both in vivo in a *P. fluorescens* subsp. *cellulosa* culture and in vitro (39, 40). This conversion occurs through modifications of the proteic and glycosidic moieties of the molecules. From this and other evidence (pH optima, composition of glycosidic fractions, and regulation patterns), it has been suggested that a single gene exists for the five extracellular cellulases and another for the membrane-bound cellulase C (36, 38).

The cellulase encoded by the gene we have cloned (14) is mostly periplasmic in *E. coli* cells (A. Lejeune, V. Dartois,

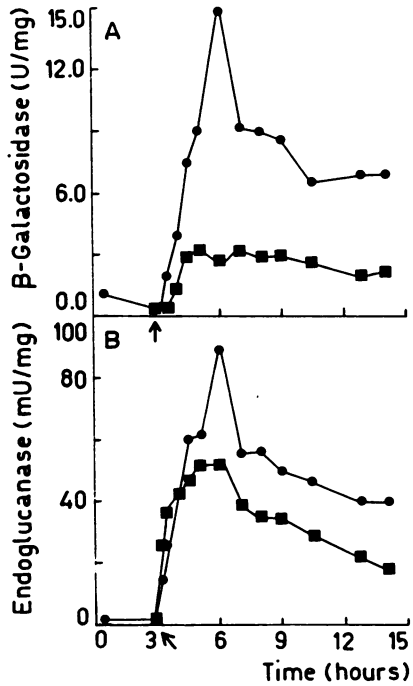

FIG. 6. Effect of glucose on enzyme synthesis by *E. coli* AKN2001(pRUCL150) grown in glycerol minimal medium. At the time indicated by the arrow (early exponential phase), β-galactosidase was induced with IPTG and 0.2% glucose was added to half of the culture. (A) β-Galactosidase; (B) endoglucanase. Symbols: ●, control culture without glucose; ■, culture with glucose.

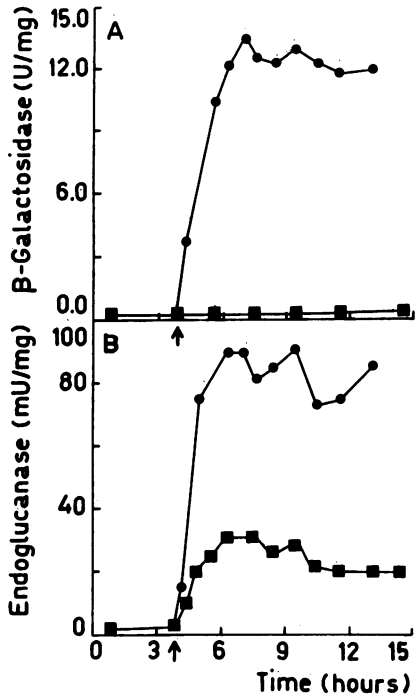

FIG. 7. Effect of cAMP on enzyme synthesis in *E. coli* CA8306(pRUCL150) grown in glycerol minimal medium. At the time indicated by the arrow (early exponential phase), β-galactosidase was induced with IPTG, and 5 mM cAMP was added to half of the culture. (A) β-Galactosidase; (B) endoglucanase. Symbols: ■, control culture without cAMP; ●, culture with cAMP.

and C. Colson, manuscript in preparation). As *E. coli* is not cellulolytic, the enzyme was not contaminated by cellulases from the host, and *E. coli* periplasmic extracts served as highly active enzyme sources without further purification. The mode of action of this cellulase is typical of an enzyme that cleaves the polysaccharide chain at random sites (Fig. 2). This mode of action and the substrate specificity define the enzyme as an endo-β-1,4-glucanase. This conclusion was anticipated, since the enzyme gives a positive result with the Congo red assay procedure (28) with CMC as the substrate. This test is based on the binding of the dye Congo red to unsubstituted cellopentaose or higher, but not lower, oligomers (34). Exoglucanases (cellobiohydrolases) can attack CMC at the nonreducing termini only until the first point of substitution is reached (10), but this does not decrease cellulose molecule chain length significantly and will not prevent dye binding. Thus, the Congo red test is specific for endoglucanases. The purified extracellular and intracellular cellulases of *P. fluorescens* subsp. *cellulosa* are also endoglucanases (37), and the cellulase encoded by another cloned gene from *P. fluorescens* subsp. *cellulosa* can be classified as an endoglucanase too, from detection of the recombinant clone by the Congo red procedure (33).

The molecular weight of the endoglucanase produced in *E. coli* was 36,000, as demonstrated by gel filtration (Fig. 1). This is in good agreement with the 40,000 and 38,000 M_r for the precursor and the mature forms of the enzyme determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Lejeune et al., in preparation) and indicates that the native enzyme is a monomer. The largest extracellular cellulase I-(1) of *P. fluorescens* subsp. *cellulosa*, from which the others are derived, is also a monomeric enzyme of M_r 200,000 (40). Its carbohydrate content is reported to represent 36% (37) or 8.4% (40) of the total mass of the enzyme. Accordingly, the proteic fraction can be calculated as M_r 128,000 or 183,000. These would be the expected molecular weights of the enzyme if its gene had been cloned into *E. coli*, since this bacterium does not glycosylate proteins. It thus appears that the gene we have cloned does not encode the extracellular endoglucanases of *P. fluorescens* subsp. *cellulosa*. In addition, the pH optimum of the extracellular endoglucanase is 8.0 (37), whereas that of the enzyme in *E. coli* was 7.0 (Fig. 3). This is identical to the pH optimum of the membrane-bound cellulase C of *P. fluorescens* subsp. *cellulosa* (37). The molecular weight of cellulase C has not been reported but can be estimated from the data presented by Yoshikawa et al. (39) to be 72,000. Taking into account its carbohydrate content of 32% (37), its proteic fraction is approximately 49,000. This is closer to, although still significantly different from, the weight we have determined for the enzyme in *E. coli*. It should be borne in mind, however, that the value of M_r 49,000 is but a rough estimate and may be relatively far from reality. A discrimination test for cellulase C or *P. fluorescens* subsp. *cellulosa* is its activity towards cellotriose (37). We have found that this oligosaccharide is not a substrate for the enzyme produced in *E. coli*. This would imply that this cellulase is not cellulase C either. However, the facts that the endoglucanase of *E. coli* is not glycosylated and that cellulase C is a glycoprotein in *P. fluorescens* subsp. *cellulosa* may be relevant here, since the glycosidic fraction of cellulases appears to play an important role in their substrate-binding properties. Indeed, the proteolytic removal of a heavily glycosylated segment of cellobiohydrolase CBHI of the fungus *Trichoderma reesei* impedes its binding to cellulose (31), and Langsford et al. (13) have reported that *Cellulomonas fimi* cellulases have a reduced ability to bind to cellulose once they are deglycosylated. A similar observation was made for an endoglucanase of *Bacteroides succinogenes* produced in *E. coli*: the enzyme did not bind to acid-swollen cellulose, and this was attributed to the lack of proper glycosylation or other posttranslational modification (27). Although these reports concern the binding of the enzymes to insoluble substrates, it cannot be totally ruled out at this stage that the endoglucanase gene we cloned in *E. coli* encodes the membrane-bound cellulase C of *P. fluorescens* subsp. *cellulosa* but that its substrate specificity has been modified by lack of glycosylation. On the other hand, the existence of at least two other cellulases in *P. fluorescens* subsp. *cellulosa*, different from cellulases I-(1) to V and C, has been reported (25, 40). They have not been characterized, and the possibility exists that the gene we cloned in *E. coli* encodes one of these cellulases. For these reasons, we suggest that this unidentified cellulase gene be designated *eglX*. To help in answering the question of the identity of the *eglX* gene, further investigations could include the preparation of antibodies against the *E. coli* enzyme and determination of their reactivity against the purified cellulases of *P. fluorescens* subsp. *cellulosa*. Alternatively, a marker exchange experiment could be designed: the cloned *eglX* gene would be insertionally inactivated with a marker gene (such as an antibiotic resistance gene) and transferred back to *P. fluorescens* subsp. *cellulosa* with a suicide vector. Homologous recombination would result in integration of the mutated allele into the chromosome, and the cellulase no longer produced, corresponding to this gene, would be identified. A similar strategy has been used to analyze a pectate lyase

gene of *Erwinia chrysanthemi* (21). No indications are available yet as to the identity of the cellulase encoded by another cloned gene of *P. fluorescens* subsp. *cellulosa* (33). However, preliminary data indicate that, if the complete structural gene has been cloned, the molecular weight of the encoded cellulase would be less than the 36,000 we report here, thus even lower than the molecular weights of cellulases I-(1) and C.

The endoglucanase described here was produced throughout the growth phase in *E. coli*. A similar rate of endoglucanase synthesis was observed in complete medium and in glycerol minimal medium in the absence of any potential inducer, indicating that this synthesis is constitutive and that the enzyme is nonadaptive. This does not imply, however, that some general mechanism of repression, such as catabolite repression, does not affect the *eglX* gene in *E. coli*. Urea is known to specifically repress genes sensitive to catabolite repression in *E. coli* (22) and represses endoglucanase synthesis in *Erwinia chrysanthemi* (3). The *eglX* gene in *E. coli* was not affected by this chemical, whereas the expression of the *lacZ* gene, measured as a control in this experiment, was significantly reduced (Fig. 5). This result should be considered cautiously, since it is not known whether urea has an effect on *P. fluorescens* subsp. *cellulosa* genes sensitive to catabolite repression. In addition, our data show that endoglucanase synthesis in *E. coli* was actually repressed by glucose (Fig. 6). This phenomenon of catabolite repression is known for many cellulases but is not general: glucose is an inducer of the endoglucanase of *Bacillus subtilis* DLG (20). In *P. fluorescens* subsp. *cellulosa*, cellulases were first considered inducible by cellulosic substances (36) but were later shown to be constitutively produced and catabolite repressed by glucose and disaccharides readily hydrolyzed to glucose by the cells (26). Our data thus confirm this second view. For *E. coli*, it is generally accepted that the intracellular concentration of cyclic nucleotides such as cAMP plays a major role in mediating catabolite repression of enzyme biosynthesis (2), although it might not be the unique regulator of catabolite repression (30). We therefore expected cAMP to be important in regulating expression of the *eglX* gene in *E. coli* and observed that endoglucanase production was markedly reduced in *E. coli* CA8306, defective in cAMP synthesis (Δ*cya*) (Fig. 7). Enzyme production was restored to a level comparable to that of the Cya^+ isogenic strain AKN2001 when exogenous cAMP was added. Yet expression of the *eglX* gene was not completely abolished in the absence of cAMP, indicating that other factors may play a role in this phenomenon. In *Thermomonospora curvata*, Wood et al. (35) showed a correlation between cellulase biosynthesis and intracellular cAMP concentration. Glucose and cellobiose caused a decline in intracellular cAMP and repressed cellulase synthesis. In a catabolite repression-resistant mutant of the same organism, intracellular cAMP levels were higher than in the wild type in both the absence and presence of repressors (9). However, the role of cAMP in the mechanism of this repression appears not to be direct, since large changes in cellulase production rates accompanied relatively small changes in cellular cAMP levels (35). Yet another direction of investigation on cellulase regulation has been opened by Shareck et al. (24), who described two endoglucanase-negative regulatory mutants of *Streptomyces lividans*. Homologous cloning of endoglucanase structural genes into these two mutants has shown that mutations at different loci affect the regulation of endoglucanase gene expression. Clearly, more studies are needed to gain a better understanding of control mechanisms involved in cellulase synthesis and may lead to manipulations to achieve greater enzyme yields.

ACKNOWLEDGMENTS

We thank J. L. Botsford for strain CA8306, M. Claeyssens for the gift of cellotriose, Y. Lobet for helping in the protein work, and G. Garot for the illustrations.

A.L. held a Research Assistant Fellowship from the Fonds de Développement Scientifique, Université Catholique de Louvain.

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