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Partial characterization of *Pseudomonas fluorescens* subsp. *cellulosa* endoglucanase activity produced in *Escherichia coli*

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SUMMARY

The recombinant plasmid, pPFC4, which carries *Pseudomonas fluorescens* subsp. *cellulosa* chromosomal DNA was previously isolated on the basis of its ability to direct the expression of endoglucanase in *Escherichia coli*. In the present study, some physical and chemical properties of this activity were characterized. The major portion (78.4%) of the endoglucanase activity is found in the periplasmic space of *E. coli*. This plasmid-encoded endoglucanase has a pH optimum of approximately 6.0 and a temperature optimum of approximately 50°C. With carboxymethylcellulose-zymograms, after polyacrylamide gel electrophoresis, periplasmic extracts from *E. coli* carrying pPFC4 show six distinct bands with endoglucanase activity. The molecular mass of the major endoglucanase band is approximately 29 kDa while the remaining bands with endoglucanase activity range from 48 to 100 kDa. Although the basis of this heterogeneity is not known, the DNA insert of pPFC4 that encodes endoglucanase activity is not large enough to contain six separate genes; hence, the observed array of endoglucanases may result from post-translational modification of one or two primary gene products.

INTRODUCTION

The hydrolysis of cellulose requires the synergistic action of three different kinds of enzymes; viz., endoglucanase, exoglucanase and β-glucosidase [4,6,7,15]. It has recently been shown for *Clostridium thermocellum* and suggested for other cellulolytic bacteria [15,16] that the cellulase enzymes are often assembled into a specialized complex which has been called a cellulosome. Current models of cellulose digestion suggest that amorphous regions of the cellulose polymer are first hydrolyzed by endoglucanases thereby exposing the ends of the cellulose chain and facilitating the subsequent removal of cellobiose, cellotriose and cellotetraose by exoglucanases [6,5].

While most of the early reports on the enzymatic

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degradation of cellulose were focussed on the fungus *Trichoderma reesei* [24], more recently a range of cellulolytic microorganisms, both fungi and bacteria, have been examined. Moreover, a large part of the current effort in this research area is directed towards the isolation and characterization of cellulase genes in addition to the study of the enzymes per se [12,13,22]. Previous studies of the cellulase enzymes in pseudomonads have indicated that endoglucanases form the major component of the cellulolytic system and that these endoglucanases occur as a number of different chromatographic forms [23,27,28,30]. As a first step towards understanding the genetic organization and regulation of these enzymes, clones expressing *Pseudomonas* endoglucanase activity in *E. coli* were recently identified [9,17,26]. The work reported here examines the endoglucanase activity in *E. coli* that is encoded by the pBR322-derived plasmid, pPFC4, which contains *P. fluorescens* subsp. *cellulosa* chromosomal DNA.

MATERIALS AND METHODS

The strain *E. coli* HB101 which contains plasmid pPFC4 (*E. coli*/pPFC4) was maintained on YT solid medium containing 40 μg/ml ampicillin [19]. In some experiments *E. coli*/pPFC4 was grown on M9 medium [19] containing 0.5 g/l casamino acids with no additional carbon source, or with 1% glucose or with 0.2% carboxymethylcellulose. *P. fluorescens* subsp. *cellulosa* (NCIB 10462) was maintained on solid Dubos salts medium to which a strip (5 × 1 cm) of Whatmann 3 MM filter paper was added as a carbon source [5].

Periplasmic and cytoplasmic fractions were prepared from *E. coli* and *P. fluorescens* subsp. *cellulosa* by the methods of Taylor et al. [25] and Yamane et al. [29], respectively. Extracellular fractions were prepared from both organisms by concentrating the spent medium, after growth of the organism, with a YM10 membrane which has a nominal molecular weight cutoff of 10 000 daltons using an Amicon 402 ultrafiltration unit at 0°C.

Endoglucanase activity was determined by measuring the amount of glucose that was released from carboxymethylcellulose as a function of time [21]. Alkaline phosphatase and β-galactosidase activities were assayed by spectrophotometrically monitoring the hydrolysis of ρ-nitrophenyl phosphate [8] and *o*-nitrophenyl-β-D galactoside [10], respectively. Protein concentrations were determined by the method of Bradford [3].

To determine the pH profile of endoglucanase activity the following buffers (final concentration 0.1 M) were used: (i) acetate from pH 4.0 to 5.5; (ii) 2[*N*-morpholino] ethanesulfonic acid (MES) from pH 5.5 to 6.0; (iii) phosphate from pH 6.0 to 7.5; and (iv) Tris from pH 7.5 to 9.0.

Polyacrylamide gel electrophoresis was performed as described by Laemmli [14]. Nondenaturing gels were run under the same conditions as denaturing gels except that SDS was omitted from both the polyacrylamide gel and the running buffer. After electrophoresis, protein bands were visualized by either silver [20] or Coomassie blue staining.

Endoglucanase activity was detected after nondenaturing polyacrylamide gel electrophoresis using a modification of the 'agarose gel overlay' technique (i.e., zymogram procedure) of Beguin et al [2]. Nondenaturing polyacrylamide gels (1.5 × 70 × 100 mm) were subjected to electrophoresis at room temperature for approximately 1 h at 100 volts, then placed onto 2% agarose gels that contained 0.1% carboxymethylcellulose. The polyacrylamide gel was covered with a glass plate and pressed down with a 100 g lead weight. After 45 min at room temperature the polyacrylamide gel was removed and the agarose gel was stained for 2-3 min with a 1mg/ml solution of Congo Red before being destained with 1 M NaCl. Zymograms from polyacrylamide gels that ranged in concentration from 5–15% were used to establish a curve of the log of the relative mobility of each band of activity against the polyacrylamide concentration [11]. A graphic comparison of the slopes of curves of proteins of known molecular weights with the various endoglucanase components was used to determine the molecular mass of each endoglucanase [11].

RESULTS

The cellular location of endoglucanase activity in *E. coli*/pPFC4 [14] cells was determined by assaying the periplasmic, cytoplasmic and extracellular fractions from mid-log cells. The bulk (94.7%) of the β-galactosidase activity is associated with the cytoplasmic fraction, most (82.3%) of the alkaline phosphatase activity is in the periplasmic fraction, and the major portion (78.4%) of the endoglucanase activity is in the periplasmic fraction (Table 1). In addition, less than 0.1% of the total endoglucanase activity was associated with the membrane fraction (data not shown). The finding that β-galactosidase and alkaline phosphatase activities are each confined to their expected cellular locations indicates that these fractions accurately reflect in vivo compartmentalization of *E. coli* proteins.

The use of the carboxymethylcellulose-Congo Red zymogram technique to examine the extracellular fraction from *E. coli*/pPFC4 indicated one band with endoglucanase activity (Fig. 1A, lane 2). By contrast, examining the periplasmic fraction indicated six bands of endoglucanase activity (Fig. 1B, lane 2). A Coomassie blue-stained band in the middle of the gel (Fig. 1B, lane 1; arrow) coincides with the major band of activity seen in the zymogram (Fig. 1B, lane 2). A band equivalent to this molecular mass is not present in the periplasmic fraction of *E. coli*/pBR322 (data not shown). The remaining bands which showed endoglucanase activity do not correspond to bands that were visualized by staining with either Coomassie blue or silver

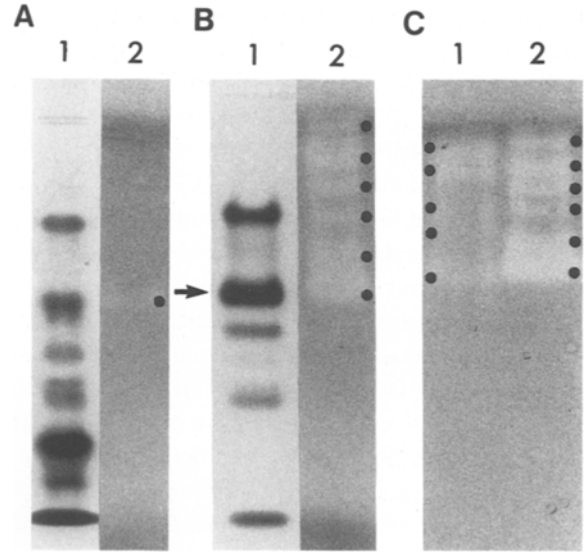

Fig. 1. Panel A: lane 1, Silver stained proteins (5 μg) of the extracellular fluid of *E. coli*/pPFC4; lane 2, Zymogram of extracellular fluid from *E. coli*/pPFC4. Panel B: lane 1, Coomassie blue stained proteins (25 μg) of periplasmic extract of *E. coli*/pPFC4; lane 2, Zymogram of a periplasmic extract of *E. coli*/pPFC4. Panel C: lane 1, Zymogram of extracellular fluid from *P. fluorescens* subsp. *cellulosa*; lane 2, Zymogram of a periplasmic extract of *E. coli*/pPFC4. The bands of endoglucanase activity are denoted by black dots. The arrow marks the Coomassie blue stained band which corresponds to the major band of endoglucanase activity.

(Figs. 1A and 1B). The molecular masses of the endoglucanases that were resolved by the zymogram technique were estimated by the method of Hedrick and Smith [11] to be approximately 29, 48, 52, 58, 48 and 100 kDa from the lowermost to the uppermost band of activity in Fig. 1B, lane 2.

Table 1

Distribution of Endoglucanase in *E. coli/pPFC4*

	Percent of Total Activity[a]		
Compartment	Endoglucanase	β-galactosidase	Alkaline phosphatase
Extracellular fluid	4.7 (9.4)	3.8 (26.0)	2.5 (0.2)
Periplasm	78.4 (180.0)	1.5 (2.5)	82.3 (2.7)
Cytoplasm	16.9 (4.9)	94.7 (41.3)	15.2 (0.1)

[a] Values in brackets indicate the specific activity in international units (U = μmol product/min/mg protein). Endoglucanase activity is presented as mU. β-galactosidase and alkaline phosphatase activities are presented as μU.

A set of five bands with endoglucanase activity is observed upon zymogram analysis of the extracellular fraction of *P. fluorescens* subsp. *cellulosa* (Fig. 1C, lane 1). A 29 kDa component is common to both *E. coli*/pPFC4 and *P. fluorescens* subsp. *cellulosa* (the lowermost bands in Fig. 1C, lanes 1 and 2).

To determine whether the multiplicity of endoglucanase bands that are observed in *E. coli*/pPFC4 periplasmic extracts reflects and association of endoglucanase with membrane fragments, *E. coli* periplasmic extracts were preincubated for 10 min at 37°C in either 0.1% SDS or 1.0% Triton X-100 and then subjected to electrophoresis in polyacrylamide gels containing the same level of detergent. Zymograms of these gels revealed that the treatment with detergent did not alter the pattern of bands (data not shown).

The bulk of the pPFC4-encoded endoglucanase activity is found in the periplasm of *E. coli*/pPFC4; whereas, in *P. fluorescens* subsp. *cellulosa* it is found primarily in the extracellular fluid [28]; therefore, the pH and temperature profiles of the endoglucanase activity from the periplasm of *E. coli*/pPFC4 and the extracellular fluid of *P. fluorescens* subsp. *cellulosa* were compared (Figs. 2 and 3). Fractions from both the extracellular fluid of *P. fluorescens* subsp. *cellulosa* and the periplasmic space of *E. coli*/pPFC4 displayed maximal endoglucanase activity at approximately 50°C (Fig. 2). The endoglucanase activity from *E. coli*/pPFC4 is somewhat more sensitive to high temperature than is the enzyme(s) produced in *P. fluorescens* subsp. *cellulosa* (Fig. 2). For example, the endoglucanase(s) synthesized in *E. coli* has less than 10% of its maximal activity at 70°C

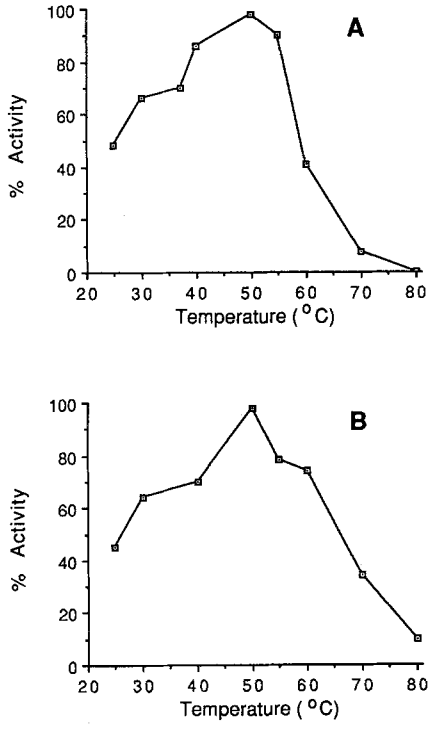

Fig. 2. Endoglucanase activity as a function of temperature. Panel A: Extracellular fluid (10 μg) from *P. fluorescens* subsp. *cellulosa* where 100% relative activity is 1150 mU of endoglucanase activity. The extract was incubated with substrate for 1 h. Panel B: Periplasmic extract (10 μg) of *E. coli*/pPFC4 where 100% relative activity is 189 mU of endoglucanase activity. The extract was incubated with substrate for 4 h. All activity measurements were made at pH 6.5.

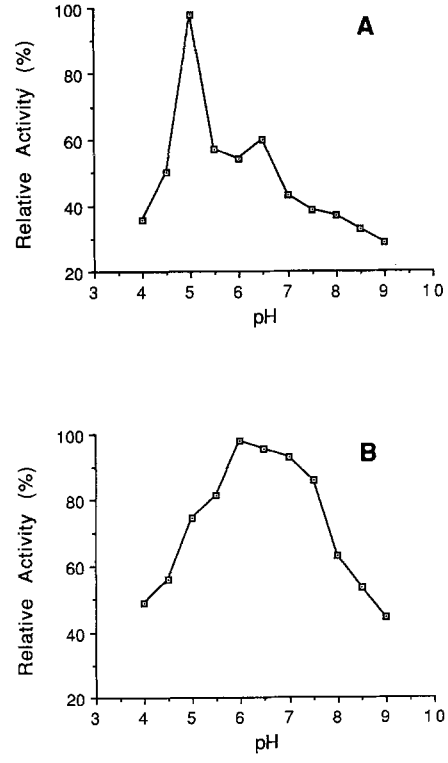

Fig. 3. Endoglucanase activity as a function of pH. Panel A: Extracellular fluid (2.5 μg) from *P. fluorescens* subsp. *cellulosa* where 100% relative activity is 2640 mU of endoglucanase activity. The extract was incubated with substrate for 1 h at 37°. Panel B: Periplasmic extract (0.5 μg) from *E. coli*/pPFC4 where 100% relative activity is 166 mU of endoglucanase activity. The extract was incubated with substrate for 4 h at 37°.

while the endoglucanase activity produced in *P. fluorescens* retains nearly 40% of its maximal activity at this temperature. The endoglucanase activity produced in *P. fluorescens* subsp. *cellulosa* displays two pH optima, one at pH 5.0 and the other at pH 6.5, while the enzyme from *E. coli*/pPFC4 shows a single pH optimum at pH 6.0 (Fig. 3).

The ability of the enzyme fractions from *E. coli*/pPFC4 and *P. fluorescens* subsp. *cellulosa* to hydrolyze a number of different celluloses was examined. It was found that: (i) the enzyme from both sources were relatively efficient at degrading carboxymethylcellulose; (ii) only the enzyme from *P. fluorescens* subsp. *cellulosa* was able to degrade crystalline celluloses such as Whatmann 3MM filter paper and alpha-cellulose; and (iii) the specific activity of the *P. fluorescens* subsp. *cellulosa* enzyme against carboxymethylcellulose was approximately 3.4-fold greater than the *E. coli*/pPFC4 enzyme.

DISCUSSION

The accumulation of $>78\%$ of the pPFC4-encoded endoglucanase activity in the periplasm of *E. coli* suggests that a precursor encoded by *P. fluorescens* subsp. *cellulosa* can be processed by *E. coli*. The major periplasmic endoglucanase component has a molecular mass of 29 000 ± 1500 daltons. DNA sequence analysis of a portion of the insert of pPFC4 revealed the presence of a putative endoglucanase gene which would code for a protein, including a presumptive leader sequence, of 32,189 daltons (Wolff et al. submitted for publication). The potential leader sequences account for approximately 1500 to 4000 daltons. Thus, the molecular mass of this open reading frame corresponds closely to the observed major acquired protein in the periplasm of *E. coli*/pPFC4. On this basis, it is assumed that the insert of pPFC4 encodes, at least, the major endoglucanase that is found in the periplasm of *E. coli*/pPFC4.

Digestion of pPFC4 with the enzyme Bal31 (data not shown) indicates that 4.6 kb of the 10.6 kb insert is sufficient to encode the multiplicity of bands showing endoglucanase activity after zymogram analysis. Since only one open reading frame has been identified within the 3.2 kb of this 4.6 kb insert that has been sequenced, pPFC4 cannot encode more than two endoglucanase genes. Thus, the multiplicity of bands that show endoglucanase activity probably reflects differences in post-translational modification of one or two encoded gene products. In this regard, Taylor et al. [25] observed that a plasmid which contained a single endoglucanase gene from *Bacteriodes succinogenes* directed the synthesis of three bands of endoglucanase activity that were localized in the periplasm of *E. coli*.

Comparisons of the temperature and pH activity profiles of endoglucanase activity from periplasmic extracts of *E. coli*/pPFC4 and from the extracellular fluid of *P. fluorescens* are consistent with the notion that some components in these extracts are present in both organisms; whereas, *P. fluorescens* has additional endoglucanase components that are not present in the *E. coli*/pPFC4 extract. For example, the temperature-activity profile of the *P. fluorescens* subs. *cellulosa* extract is broader than the profile for the *E. coli*/pPFC4 extract. Similarly, the pH-activity profile of the *P. fluorescens* subsp. *cellulosa* extract exhibits two distinct optima while the *E. coli*/pPFC4 extract shows only one. Both the earlier studies at the protein level [28] and the more recent data that have been obtained from gene cloning experiments [9,17,26] are consistent with the notion that *P. fluorescens* subsp. *cellulosa* contains a family of endoglucanases encoded by different genes and, as well, that some of these endoglucanases are differentially post-translationally modified. Based on our observations, pPFC4 encodes at least one and probably no more than two members of this family of endoglucanases. The genes isolated by Lejeune et al [17] and Gilbert et al [9] encode other members of this gene family. In addition, the endoglucanase(s) that are encoded by pPFC4 is kinetically and physically distinct from the *P. fluorescens* subsp. *cellulosa* endoglucanase that has been characterized by Lejeune et al [18]. Plasmid pPFC4 encodes an endoglucanase with a pH optimum of 6.0 and a molecular weight of 29 kDa while the enzyme characterized by Lejeune et al., [18] has a pH optima of 7.0 and a molecular weight of 36 kDa.

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