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Bacillus subtilis β-1,4-Endoglucanase Products from Intact and Truncated Genes Are Secreted into the Extracellular Medium by *Escherichia coli*†

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We compared the secretion of a *Bacillus subtilis* endo-β-1,4-glucanase (EC 3.2.1.4) in *B. subtilis* and of the product from the cloned gene (pC6.3) expressed in *Escherichia coli*. The cloned enzyme has been isolated previously as the 52.2-kilodalton (kDa) species predicted from the gene sequence (R. M. MacKay, A. Lo, G. Willick, M. Zuker, S. Baird, M. Dove, F. Moranelli, and V. Seligy, Nucleic Acids Res., 14:9159–9170, 1986); this 52.2-kDa species is then converted to an active 35.8-kDa species. The 35.8-kDa species has a segment removed from the COOH terminus. Endoglucanase products were identified by use of an antibody directed to the 35.8-kDa enzyme. Time course studies of the secretion in *B. subtilis* showed that the enzyme was first secreted as a 52.2-kDa proenzyme. This was cleaved progressively to a product of about 32 kDa. Time course analysis of the expression of the cloned product from pC6.3 in *E. coli* showed that about 70% of the endoglucanase activity was found extracellularly. Analysis of active products from three deletion clones showed that the expression pattern of the endoglucanase was not affected by removal of the transcription termination signal and that neither expression nor secretion was substantially altered by removal of a region coding for up to 163 residues of the carboxyl terminus.

Protein excretion into the culture medium is desirable in biotechnology in order to facilitate recovery of the product. Many heterologous proteins, when cloned in *Escherichia coli*, are secreted into the periplasmic space when the signal peptide is present in the gene insert (3). A few proteins are excreted further into the medium. A cloned penicillinase of an alkalophilic *Bacillus* species (6, 7, 9) and a cloned protease of *Serratia marcescens* (24) have been reported to be excreted into the medium from *E. coli* cells. In the former case, the excretion appeared in the late stationary phase and was accompanied by secretion of the host periplasmic enzymes (6, 7). In the case of the cloned protease, it was suggested that the removal of the NH_2-terminal peptide and a large COOH-terminal fragment were both required for the excretion of the mature protease (24). Very recently, a cloned xylanase from an alkalophilic *Bacillus* species has been reported to be secreted through the outer membrane of *E. coli* (4).

Recently, we have cloned and sequenced the endoglucanase gene of *Bacillus subtilis* PAP115 and expressed it in *E. coli* (12, 21). This gene encodes a protein of 499 amino acid residues (M_r = 55, 234) and possesses a typical *B. subtilis* signal peptide. The endoglucanase was found to be excreted into the medium from *E. coli* cells (12). It was observed that a 35.8-kilodalton (kDa), stable, enzymatically active species could be isolated. This species was apparently identical to the 52.2-kDa enzyme that was expected after removal of a signal peptide, but it lacked a 163-amino-acid sequence at the COOH terminus (12).

Here we report results of a study of the secretion patterns of the endoglucanase in *B. subtilis* and *E. coli*. We show that the native *B. subtilis* endoglucanase is secreted directly into the medium and is then cleaved extracellularly to give an enzymatically active form of about 32 kDa. The cloned endoglucanase, when expressed in *E. coli*, is processed similarly. Cleavage appeared to occur in the periplasm, prior to excretion into the medium. The final active product was found to be slightly larger than that which was formed in *B. subtilis*. Additionally, a deletion clone with about one-third of the COOH terminus removed was found to be efficiently excreted from *E. coli*.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* PAP115 was the source of the endoglucanase used in this study (21). Cloning was carried out in *E. coli* JM103 [Δ(*lac pro*) *thi strA supE endA sbcB15 hsdR4* F′ *traD36 proAB lacI*^{q} ZΔM15).

Construction and subcloning of the deletion clones. The preparation of deletion clones has been described previously (21). The constructions of those clones used in this study are summarized in Fig. 1. Plasmid pC6.3 contained the entire structural gene plus 5′- and 3′-untranslated regions containing the promoter and transcription terminators, respectively (12). A 3.1-kilobase-pair *Hin*dIII fragment from pC6.3 subcloned into M13mp9 (14) generated a clone (SBO) that lacked the *B. subtilis* transcription terminator. *Bal* 31 digestion of pC6.3 at the 3′ end of the *Pst*I fragment, followed by subcloning into M13mp8 (*Pst*I-*Hin*cII sites), yielded a set of clones with deletions at the COOH terminus of the endoglucanase (21). Recombinant phage C1-1 was derived from the M13mp8 subclone C1 by excision of its insert with *Pst*I and

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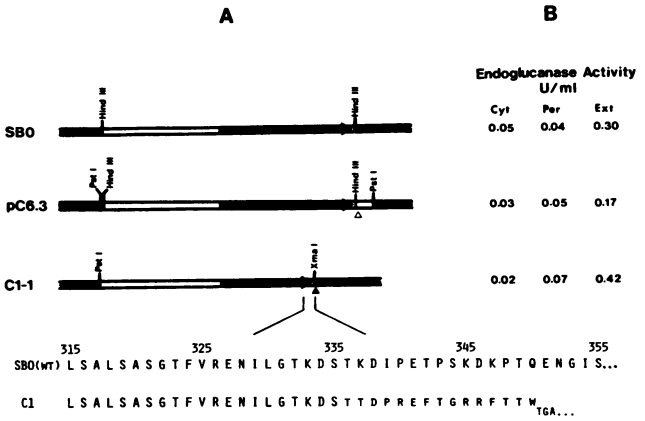

FIG. 1. Construction of clones and endoglucanase activities of their products. (A) Construction of clones pC6.3, SBO, and C1-1. The lengths of the endoglucanase sequence are indicated by closed arrows. Open boxes indicate the flanking regions from pC6.3. Plasmid pC6.3 retained the 3'-flanking region which contains a putative [*rho*]-independent transcription termination sequence (12). The transcription termination sequence (△) is absent in SBO and C1-1. The translation stop codon in C1-1, which is found in the M13mp9 sequence, is indicated (▲). Hatched boxes represent the pUC8 vector sequence, while dotted boxes represent the M13mp9 phage sequence. The COOH-terminal sequence (one-letter code) derived from the DNA sequence of deletion construct C1-1 is shown below to the position of the nearest stop codon in the flanking sequence of M13mp9 (small letters). The corresponding sequence in the full endoglucanase is shown above it for comparison. The nucleotide sequences (large letters) are derived from the *Hin*cII-*Xma*I sequence of M13mp8 and the *Xma*I sequence of M13mp9 (see text for further details). (B) Endoglucanase activities in cytoplasmic (Cyt), periplasmic (Per), and extracellular (Ext) fractions for each clone after 6 h of growth are expressed as units per milliliter of bacterial culture.

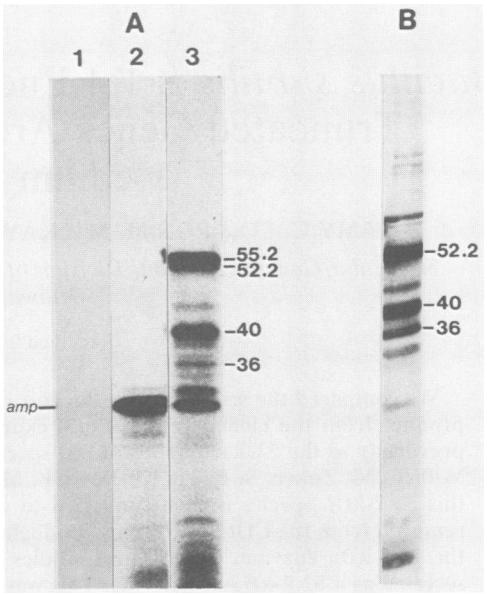

FIG. 2. Clone-directed products. Autoradiograph of products from transformed maxicells labeled with L-[^{35}S]methionine and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10 to 20% acrylamide gradient). (A) Products were from *E. coli* CSR603 (lane 1), *E. coli* CSR603(pUC8) (lane 2), and *E. coli* JM103(pC6.3) (lane 3). (B) Western blot of cytoplasmic proteins (24 h) from *E. coli* JM103(pC6.3), detected with anti-35.8-kDa endoglucanase, followed by detection with ^{125}I-labeled protein A, which is shown for comparison. Numbers to the right of the lanes are molecular masses (in kilodaltons).

*Xma*I and ligation into the corresponding sites of M13mp9. Phage C1-1 expresses a shortened version of the endoglucanase in *E. coli* JM103. It has been sequenced and has been found to terminate at nucleotide 1738 (codon 336) of the parent sequence (12). It retains the original *B. subtilis* promoter, but it lacks the translation stop codon. Translation should continue within M13mp9 until it reaches the stop codon TGA (Fig. 1).

Maxicell labeling. *E. coli* CSR603 was transformed with either pC6.3 or pUC8 by using the calcium chloride procedure (13). The transformed CSR603 strains were then irradiated at 4.8 ergs/mm^2 into a 10-ml culture for 25 to 26 s and were subsequently labeled in Hershey minimum medium (23) for 1 h at 37°C with 24 μCi of L-[^{35}S]methionine, as described by Sancar et al. (19). The radioactive products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11) and autoradiography.

Growth and fractionation of *E. coli* and *B. subtilis* cells. Cells of *E. coli* JM103(pUC8) or JM103(pC6.3) were cultured in Luria broth with ampicillin (35 μg/ml) at 37°C. Portions (5 ml) of the culture were withdrawn every hour until the cells reached the stationary phase. The fractionation of extracellular, periplasmic, membrane, and cytoplasmic endoglucanases was carried out by the method of Cornelius et al. (1).

The overnight culture (1%) of *B. subtilis* was innoculated into 200 ml of B broth, which contained 1% tryptone, 0.8% NaCl, and 0.0001% thiamine (14). The culture was incubated at 37°C with vigorous shaking. Portions (5 ml) were withdrawn at 1, 2.5, 3.5, 5, 7, 11, and 24 h. The cytoplasmic fraction was obtained by washing the cell pellet twice with 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA, and then the cells were suspended in 0.2% lysozyme at room temperature for 30 min. The cell debris was removed by centrifugation; and the supernatant, cytoplasmic, and extracellular fractions were used for subsequent analyses.

Enzyme assays. Endoglucanase activities were determined by the reducing sugar method (15). One unit of activity was defined as the amount of enzyme which liberated 1 μmol of reducing sugar, estimated as glucose equivalent, per minute at 37°C.

Alkaline phosphatase activities were measured by the method of Schlesinger and Barrett (20).

Immunodetection of endoglucanase in polyacrylamide gels. Western blots were carried out as described by Towbin et al. (22) by using rabbit antibody to the 35.8-kDa endoglucanase from *E. coli* JM103(pC6.3). The antisera were precipitated with 50% ammonium sulfate before use.

RESULTS AND DISCUSSION

Products from plasmid pC6.3. The number and size of proteins encoded by the 5.9-kilobase plasmid pC6.3 were determined by the Maxicell technique (19). Several labeled proteins were observed on analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2). Two major ones had molecular masses of 55.2 and 52.2 kDa, respectively. These presumably correspond to the full-length endoglucanase before and after signal peptide cleavage, respectively. A Western blot of products from the cytoplasm of *E. coli* JM103(pC6.3), which were detected with the anti-35.8-kDa endoglucanase, showed a molecular weight distribution very similar to those of the Maxicell-labeled

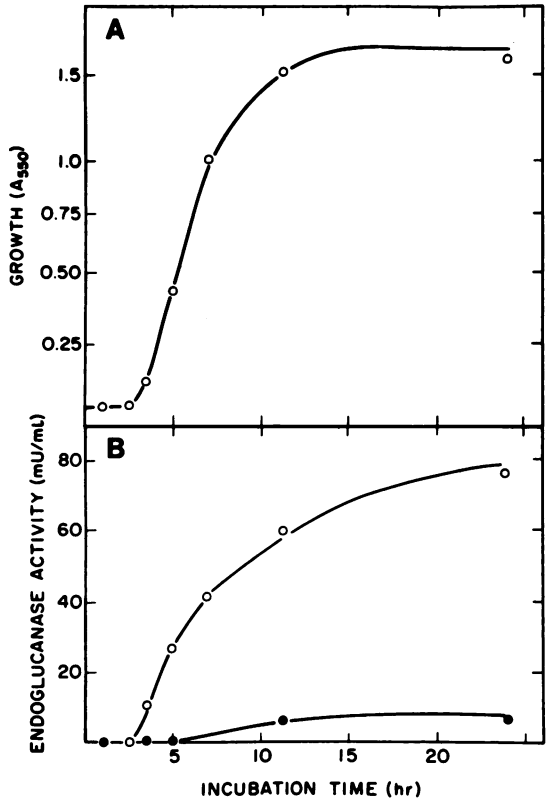

FIG. 3. Time course of endoglucanase production by *B. subtilis* PAP115. Growth (A) and endoglucanase activities (B) in the extracellular (○) and cytoplasmic (●) fractions were measured for cultures growing at 37°C. Endoglucanase assays were performed by the reducing sugar method (16) and are expressed as microunits per milliliter of bacterial culture.

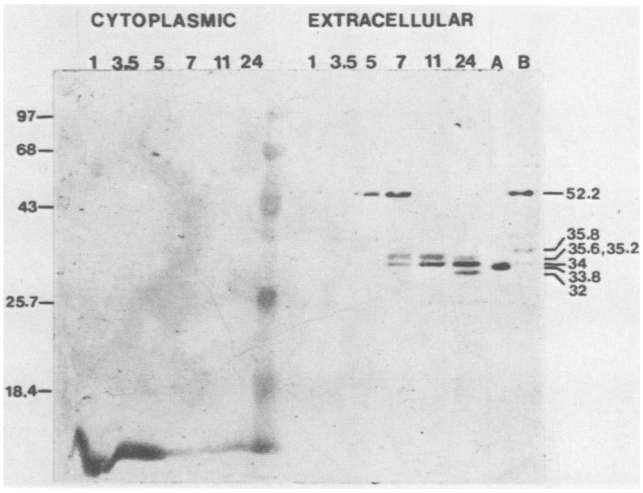

FIG. 4. Localization of endoglucanase from *B. subtilis* PAP115. Portions were withdrawn from the cell culture at the indicated times (in hours) and fractionated as describe in the text. The Western blot of these fractions, after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was reacted with the anti-35.8-kDa endoglucanase and then was reacted with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG). The values for the molecular mass markers (center lane) are shown on the left (in kilodaltons). Extracellular endoglucanase products from M13mp9 (C1-1) (lane A) and plasmid pC6.3 (lane B) are also shown. The molecular masses for the smaller forms on the right (in kilodaltons) were estimated relative to an assumed value of 35.8 kDa for the *E. coli* low-molecular-mass species.

products (Fig. 2B). An exception was the apparent absence of the 55.2-kDa endoglucanase with the signal sequence intact. We observed relatively little cross-reaction with *E. coli* cytoplasmic proteins (e.g., see Fig. 7). The prominent 40-kDa species seen in Fig. 2 was likely a degradation product that accumulated in the cytoplasm and that was not secreted. Thus, most of the proteins observed in this maxicell experiment were accounted for by the endoglucanase, after signal sequence cleavage, and proteolytic degradation products.

Time course of the expression and secretion of endoglucanase in *B. subtilis* and *E. coli*(pC6.3). Almost all of the endoglucanase secreted by *B. subtilis* was secreted into the culture medium (Fig. 3). The endoglucanase initially was secreted as a 52.2-kDa protein. This secretion appeared to be coincidental with cell growth (Fig. 3 and 4). Progressive cleavage of the 52.2-kDa enzyme yielded products with estimated molecular masses of 35.6, 35.2, 34, and 32 kDa (Fig. 4). Activity was retained, despite the progressive appearance of smaller proteins during and after the log phase. These smaller proteins likely have portions of their COOH termini removed in a manner analagous to that described below for the cloned endoglucanase that is expressed in *E. coli*.

We observed very little enzyme activity or antibody-detectable protein in the cytoplasmic fraction of *B. subtilis* (Fig. 4). It thus appears that the enzyme is directly transported through the cell membrane following its synthesis. Alternatively, nontransported endoglucanase must be degraded rapidly, since the cytoplasmic level was too low for antibody detection.

The cloned product in *E. coli* was handled similarly, but not identically, to that described above for *B. subtilis*. As much as 70% of the endoglucanase activity was found in the extracellular fraction (Fig. 5). In contrast to the observation given above, about 25% of the activity of the cloned endoglucanase from *B. subtilis* IFO3034 was found in the extracellular fraction of *E. coli* (8). Even less (about 1%) has been found to be extracellular with cloned endoglucanases from either *B. subtilis* DLG (17) or *Clostridium thermocellum* (2). Most of the endoglucanase activities in *E. coli* (8), *B. subtilis* DLG (17), and *C. thermocellum* (2) were found in the periplasm or were retained inside the cytoplasmic membrane. Since all of these studies used a similar method of cell fractionation (the osmotic shock method for obtaining the periplasmic fraction), the differences are the result of an intrinsic property of either the endoglucanase protein or the host.

The property of this endoglucanase which gives rise to its excretion through the outer membrane of *E. coli* is not known. It is clear from our results, however, that the COOH terminus portion of the molecule is not involved, since both the 52.2- and 35.8-kDa species were excreted from the periplasmic space. In addition, the product from a deletion clone was excreted from *E. coli*, and this clone expressed a product devoid of about one-third of the COOH terminus (see below). These results are in contrast to those of the *Serratia* serine protease, for which it has been suggested that the COOH terminus is involved in its extracellular secretion from *E. coli* (24). An alkalophilic *Bacillus* sp. penicillinase

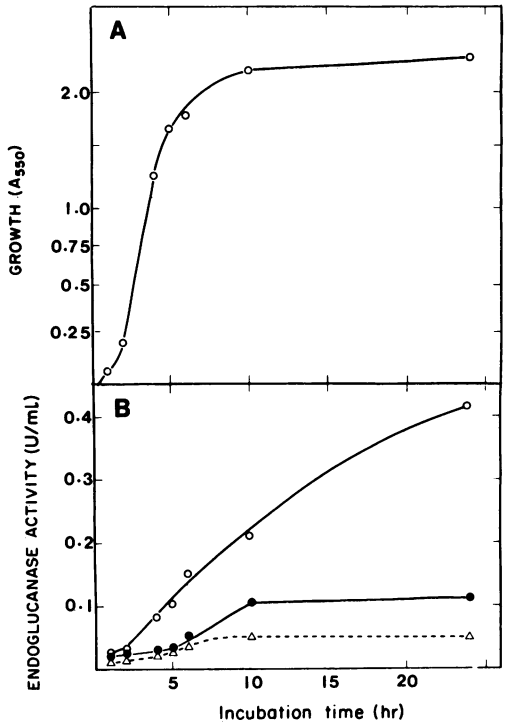

FIG. 5. Time course of endoglucanase production by *E. coli* JM103(pC6.3). Growth (A) and endoglucanase activities (B) in the extracellular (○), periplasmic (●), and cytoplasmic (△) fractions for cultures grown at 37°C.

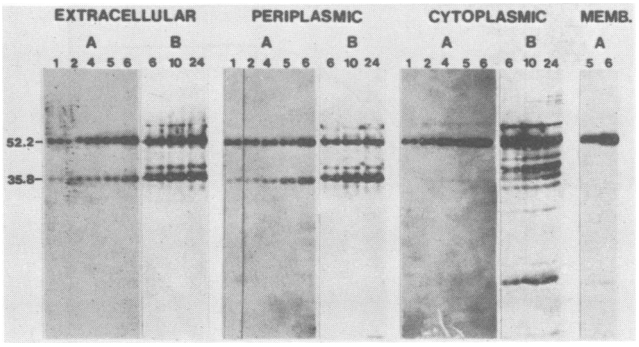

FIG. 6. Localization of endoglucanase from *E. coli* JM103 (pC6.3) as a function of time. At the indicated times (in hours), portions were removed from the culture and fractionated as described in the text. After reaction with the anti-35.8-kDa endoglucanase, Western blots were reacted with either ^{125}I-labeled protein A (A) or alkaline phosphatase coupled to anti-rabbit IgG (B). Loadings (expressed as microliters per milliliter of bacterial culture) are extracellular (20 μl/ml), periplasmic (100 μl/ml), and membrane (100 μl/ml) in panel A and extracellular (20 μl/ml), periplasmic (40 μl/ml), and cytoplasmic (40 μl/ml) in panel B. Note that the two detection methods overlapped at 6 h. Numbers to the left are molecular masses (in kilodaltons).

(9) has been reported to be excreted but, in addition, to be accompanied by a significant leakage of the host periplasmic enzymes into the culture medium. It is unlikely that the observed activity in the culture medium is due to leakage or contamination from the periplasm during the fractionation of the cells. The distribution of alkaline phosphatase, a periplasmic marker, was measured concurrently with the cellulase expression. A total of 85 to 90% of its activity was found in the periplasmic fraction, and only 10 to 15% of its activity was found in the cytoplasmic fraction. β-Galactosidase, a cytoplasmic marker, was observed to be retained within the cytoplasmic fraction. We also observed that the endoglucanase could not utilize the cell wall of *E. coli* as a substrate. Again, there was no evidence for leakiness of the host *E. coli* JM103 outer membrane as an explanation for the excretion of the cloned endoglucanase. The common structure, if any, that brings about this excretion is not yet clear. It is possible that some structural element that is common to *E. coli* proteins which are secreted into the periplasm but not into the medium is absent from these foreign excreted proteins.

The endoglucanase was also cleaved to an active lower-molecular-weight species in *E. coli*, as was observed in *B. subtilis*. However, in this case only one principal component, with a molecular mass of 35.8 kDa, was observed (Fig. 6). There was very little enzyme activity in the cytoplasmic and membrane fractions. The species excreted from *E. coli* and *B. subtilis* were not the same size, suggesting that the processing proteases in the two organisms have different specificities. It also implies a considerable flexibility in the degree to which the COOH terminus can be processed and still result in an active enzyme.

Our 35.8-kDa species apparently corresponds to the 35.2-kDa cell-associated endoglucanase produced from the *B. subtilis* DLG cloned endoglucanase gene (18). In addition, Robson and Chambliss (18) have found another active component inside the *E. coli* cells which has an estimated molecular mass of 51 kDa (17).

The processing of the 52.2-kDa protein could occur either in the cytoplasm or in the periplasmic space, where many proteolytic enzymes are located (5). The observed progressive appearance of the lower-molecular-weight form in the periplasm suggests that the proteolytic cleavage occurs there (Fig. 6). Not all of the 52.2-kDa proenzyme was processed prior to excretion. This is clear from the relative concentration of the 52.2- and 35.8-kDa proteins associated with the extracellular and periplasmic fractions, respectively. Thus, the excretion of the 52.2-kDa protein from the periplasm to the culture medium is likely just as efficient as that of the 35.8-kDa enzyme.

Distribution and size analysis of products from gene constructs expressed in *E. coli*. The enzyme activities in the cellular fractions of the *E. coli* transformants are shown in Fig. 1. A Western blot of these fractions is shown in Fig. 7. The removal of the transcription termination signal of the parent gene in clone SBO did not affect the expression or secretion of the endoglucanase significantly. The deletion clone C1-1, which produces a 33.8-kDa product, secreted most of its product extracellularly. These products are therefore near the maximum deletion from the COOH terminus that is possible before loss of enzymatic activity occurs. This suggests that the presence of about one-third of the COOH terminus of the protein has no physiological significance, in *E. coli* at least, in terms of its secretion. Nonetheless, it could have a function in *B. subtilis*. One possibility would be a role in the correct folding of the protein during biosynthesis. The correct folding of deletion clone C1-1 suggests that this is an unlikely possibility. It is possible that the 52.2-kDa initial product is more stable in the cytoplasm or more facilely excreted from *B. subtilis* than are the smaller forms. We have recently purified the various species and are examining their enzymatic and physical properties (K. Laderoute, A. C. Lo, M. Hefford, V. L. Seligy, and G. E. Willick, manuscript in preparation).

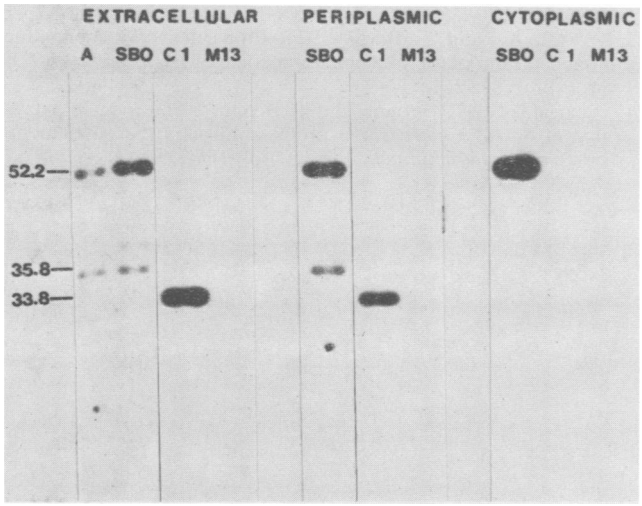

FIG. 7. Endoglucanase produced by deletion clones C1-1 derived from pC6.3. Overnight cultures of *E. coli* JM103 infected with SBO and C1-1 were innoculated into 10 ml of LB broth, which contained 1% tryptone, 0.5% yeast extract, and 0.5% NaCl (pH 7.5), and were grown for 6 h at 37°C. The cultures were separated into extracellular, periplasmic, and cytoplasmic fractions and were analyzed as described in the legend to Fig. 6. *E. coli* JM103 infected with M13mp9 was used as a control. Loadings (expressed as microliters per milliliter of bacterial culture) are extracellular (20 μl/ml) periplasmic (100 μl/ml), and cytoplasmic (100 μl/ml). Antibodies were detected with ^{125}I-labeled protein A. The extracellular endoglucanase product from pC6.3 is also shown (extracellular, lane A). Numbers to the left are molecular masses (in kilodaltons).

Hydropathy of 52.2-kDa endoglucanase. A plot of the hydropathic index of a protein as a function of amino acid residue can provide useful information on regions which might be very accessible to solvent and as well to proteolytic attack. Such a plot is shown in Fig. 8, which was done by the method of Kyte and Doolittle (10). The COOH terminus region was substantially more hydrophilic than the rest of the molecule, a result which is consistent with the fact that it is largely on the surface of the 52.2-kDa molecule. Thus, it certainly could be expected to be the most susceptible to proteolytic attack.

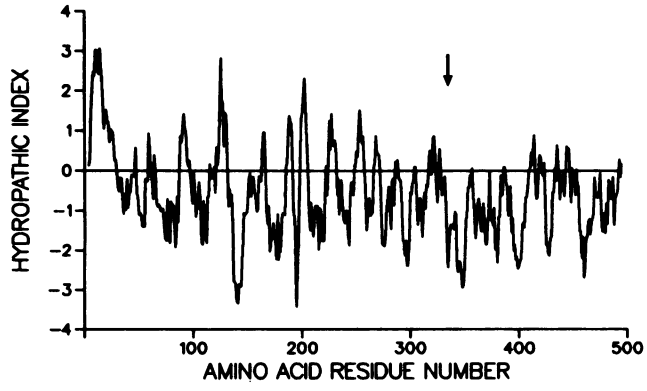

FIG. 8. Hydropathy of *B. subtilis* PAP115 endoglucanase. The values were calculated from the derived amino acid sequence (12) by the method of Kyte and Doolittle (10). Values were averaged over seven amino acids. The C1-1 deletion site is indicated by an arrow.

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