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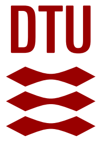

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# Expression of a *celE* Gene from *Clostridium thermocellum* in *Bacillus*

RUSTEM I. AMINOV,[1]* NIKOLAI P. GOLOVCHENKO,[2] AND KUNIO OHMIYA[1]

*Faculty of Bioresources, Mie University, Tsu 514, Japan[1] and Institute of Biochemistry & Physiology of Microorganisms, Russian Academy of Sciences, Puschino, 142292, Russia[2]*

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**The level of expression of the *Clostridium thermocellum celE* gene in the asporogenous *Bacillus subtilis* strain 1A718 did not exceed the endogenous background level. However, when transformed into sporogenous strains, *celE*-containing constructs allowed the cells to express a high level of thermostable carboxymethylcellulase (CMCase) activity which was detected exclusively in the culture supernatant. The sporulation efficiency was impaired in the *celE*-carrying strains. Most of the thermostable CMCase activity in the recombinant strains was attributed to the stationary phase of growth, and production of the enzyme could be further enhanced by increasing the cultivation temperature from 37°C to 42°C. Even when expressed in an extracellular proteases deficient mutant, the protein product was cleaved in the P-T-rich linker sequence (2 sites) and at a site downstream of the putative signal peptidase recognition site. As a consequence, the enzymatically active protein could be isolated only in a truncated form. Plasmid pHE9102, the *celE*-containing construct, undergoes significant structural rearrangements in *Bacillus stearothermophilus* strains, preventing any detectable expression.**

[**Key words:** *Clostridium thermocellum, Bacillus, celE*, expression]

The substantial progress in biochemistry and molecular biology of bacterial cellulases and xylanases during the past decade has been possible mainly due to the exploitation of the well-established molecular cloning system of *Escherichia coli*. At the same time, interest was increasing in heterologous expression of these hydrolytic enzymes in microorganisms other than *E. coli* and the numerous examples of prospective hosts for such experiments include yeasts (1–3), lactic acid bacteria (4–6), thermophilic bacteria (7, 8), ethanol-producing bacteria (9, 10), and bacilli (7, 11, 12). Other interesting approaches are the heterologous expression of a bacterial endoglucanase gene in mammalian cells (13) and in transgenic mice (14). These studies are not only intrinsically interesting but have suggested applications with undoubted commercial potential.

The safety of *Bacillus subtilis*, its ability to secrete large amounts of protein and its well-developed genetics and molecular cloning techniques make it an attractive host for expression of heterologous gene products. In particular, in the field of heterologous expression of cellulase and xylanase genes from the thermophilic anaerobic bacterium *Clostridium thermocellum*, bacilli are probably the most taxonomically closely related (15) and genetically well-developed systems available for expression studies. In our study, an endoglucanase gene of the thermophilic anaerobic bacterium *C. thermocellum*, *celE*, was used as a model gene for heterologous expression in *B. subtilis* and in thermophilic bacilli. Two plasmids with different copy numbers in *B. subtilis* cells, pUB110 (high) and pHPS9 (low), were used for cloning experiments. In this paper we report the data we obtained on expression of the *celE* gene in bacilli and on purification of the protein product.

* Corresponding author. Present address: STAFF-Institute, 446–1 Ippaizuka, Kamiyokoba, Tsukuba, Ibaraki 305, Japan.

## MATERIALS AND METHODS

**Strains and plasmids** Strains and plasmids used in this work are listed in Table 1.

**Media and growth conditions** *B. subtilis* strains were grown on LB medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 1 *l* of distilled water) and *Bacillus stearothermophilus* strains on BST medium (16) which were solidified, if necessary, with 1.8% agar. Periodic cultivation of bacilli was performed under aerobic conditions in 100 ml of the appropriate media with vigorous agitation at 37°C (mesophilic strains) or at 55°C (thermophilic strains). Growth was monitored turbidometrically at 660 nm. Antibiotics for selection of transformants and maintenance of plasmids were added at the concentration of 10 $\mu$g/ml (chloramphenicol, kanamycin and erythromycin).

**Sporulation efficiency determination** The efficiency of sporulation of *B. subtilis* strains was determined by plating serial dilutions of a 24 h culture of cells grown in Schaeffer sporulation medium (16) with the appropriate antibiotic. Cells were plated before and after treatment with 0.1 volume chloroform to obtain a viable cell count and a spore count.

**Electrotransformation procedure** Electrotransformation of bacilli strains was performed as described by Kusaoke *et al.* (17). Electroporation was performed using an electroporation device from Shimadzu (Kyoto), generating square-waveform pulses. Plasmid DNA (0.1 $\mu$g) in 1 $\mu$l of sterile distilled water was added to 9 $\mu$l of cell suspension immediately before electric pulse application. The electric field strength was fixed at 12 kV/cm. Each sample was regenerated in the appropriate broth at 37°C or 55°C for 4 h before plating onto the selective media.

**Plasmid DNA isolation** Plasmid DNAs from *B. subtilis* and *E. coli* were isolated by a modified alkaline lysis procedure (18) and from *B. stearothermophilus* strains by a mini-prep method developed for lactic acid bacteria (19). DNA fragments from the agarose gels

TABLE 1. Strains and plasmids used in this work

| Strain, plasmid | Properties | Source/reference |
|---|---|---|
| *B. subtilis* 168 | *trpC2* | Laboratory stock |
| *B. subtilis* 1A718 (pHPS9R) | *his met rib trpC2 tyr ura* $r^-$ $m^-$ *lacZ*Δ*M15* $Km^R$ *spo* | BGSC[a] |
| *B. subtilis* 1A751 | *aprE nprE bglT/bglS* Δ*EV eglS*Δ*102 his* | BGSC |
| *B. stearothermophilus* 9A2 | prototrophic | BGSC |
| *B. stearothermophilus* 9A5 | prototrophic $Rif^R$ *hsrl hsml* | BGSC |
| *E. coli* JM109 | F′ *traD36 lacI*$^q$ Δ*(lacZ)M15 proA*$^+$*B*$^+$/*te*14$^-$(McrA$^-$) Δ*(lac-proAB) thi gyrA96* ($Nal^r$)*endA hsdR17* ($r_k^-m_k^+$) *relA1 supE44 recA1* | Stratagene |
| pUB110 | $Km^R$ | Laboratory stock |
| pUCE102 | $Ap^R$ *celE* | (20) |
| pUB102 | $Km^R$ *celE* | This work |
| pHPS9 | $Em^R$ $Cm^R$ *lacZ′* | BGSC |
| pHE9102 | $Em^R$ $Cm^R$ *lacZ′ celE* | This work |

[a] BGSC—Bacillus Genetic Stock Center.

were purified using the GENECLEAN II kit (BIO 101 Inc., La Jolla, USA).

***In vitro* DNA manipulations** All routine molecular cloning procedures were carried out essentially as described in the handbook by Sambrook *et al.* (18). Restriction and DNA modification enzymes, chemicals and reagents for molecular biology experiments were obtained from Boehringer Mannheim Japan (Tokyo), Serva (Heidelberg, Germany), Sigma (St. Louis, USA) and Takara Shuzo (Kyoto).

**Enzyme activity** Thermostable CMCase activity in the culture supernatants and in the crude cell extracts and qualitative MUCase activity on plates were determined as described earlier (20). One unit of CMCase activity was defined as the release of 1 μM of glucose equivalent from CMC per minute at 60°C.

**SDS-PAGE and zymograms** SDS-PAGE was performed in 12% resolving gels (21). CMCase zymograms were prepared by including 0.2% CM-cellulose into the SDS-PAGE resolving gel with subsequent visualization with Congo Red (22).

**Purification of EGE** Endoglucanase E was isolated from a 12 h culture of recombinant *B. subtilis* grown in LB medium in the presence of the appropriate antibiotic. Cells were removed by centrifugation from the culture supernatants of recombinant bacilli strains and the extracellular proteins were precipitated with ammonium sulfate at 45% saturation. The precipitate was dissolved in the corresponding buffer (50 mM phosphate buffer, pH 7.0; 100 mM phosphate-citrate buffer, pH 6.4; or 25 mM Tris-HCl buffer, pH 7.5) with or without salt (NaCl, $(NH_4)_2SO_4$ or $Na_2SO_4$) (Table 4), and excess ammonium sulfate was removed by ultrafiltration. Thus, several combinations of buffers and salts were tested for determining the optimal purification procedure using a gel-filtration column. Samples were applied to the prepacked preparative gel-filtration column Superdex 200 from Pharmacia (Uppsala, Sweden), equilibrated with the same buffer-salt combination and connected to an FPLC system from Pharmacia. Five ml fractions were collected, followed by change of the buffer, desalting and concentration of samples using an ultrafiltration unit from Amicon (USA).

The magnitude of retardation of protein elution during gel-filtration was expressed in terms of the capacity factor, $k$, determined as

$$k = V_{exp} - V_{theor}/V_{theor}$$

where $V_{exp}$ and $V_{theor}$ are, respectively, the elution volumes obtained in an experiment and calculated according to the molecular weights data. The latter value was calculated from the calibration curve for the Superdex column as a function of protein molecular weight using molecular weight standards from Serva (Heidelberg, Germany): DNP-L-alanine, cytochrome C, chymotrypsinogen A, ovalbumin, and bovine serum albumin.

**Protein sequencing** EGE purified from different recombinant strains was fractionated by SDS-PAGE and electroblotted onto Immobilon™-PSQ PVDF membrane (Nihon Millipore Ltd., Tokyo). The N-terminal sequence was determined by automated Edman sequencing using a 470 gas-phase sequencer (Applied Biosystems Inc. Japan, Tokyo).

**Immunological procedures** Polyclonal antibodies against EGE were raised in mice according to the standard protocol (18). Western blotting was performed using the BM Chemiluminescence Western Blotting Kit (Mouse) under the conditions recommended by the supplier (Boehringer Mannheim GmbH, Mannheim, Germany).

## RESULTS

**Construction of recombinant plasmids for expression in bacilli** The *celE* gene of *C. thermocellum* was initially cloned on the *E. coli-B. subtilis* shuttle vector pMK4 and the resulting construct, pCE102, was characterized by a high level of structural instability in *B. subtilis* 168 cells (23). This might be due to a lethal effect of overexpression of the gene under the control of its own regulatory sequences as has been shown for some other *cel* genes of *C. thermocellum* (7). Thus, we chose a strategy allowing us both to select recombinant clones which express the *celE* gene under the control of its own promoter and to adjust its coding sequence under the control of the internal promoter (24) of the cloning vector pUB110 (Fig. 1). The recombinant plasmid pUCE102 was linearized by cleavage at the SalI site and treated with exonuclease Bal31 to generate deletions in the 5′-terminal region of the *celE*-containing DNA fragment. After blunting by Klenow enzyme, a second cut was made at the BamHI site of the polylinker region of the vector downstream of the 3′-terminus of the *celE* gene. The resulting mix of the truncated *celE*-carrying fragments was isolated from the preparative gel and ligated into the vector pUB110 linearized by PvuII-BamHI digestion. The constructs expressing the highest thermostable CMCase activity were analyzed and in all of them

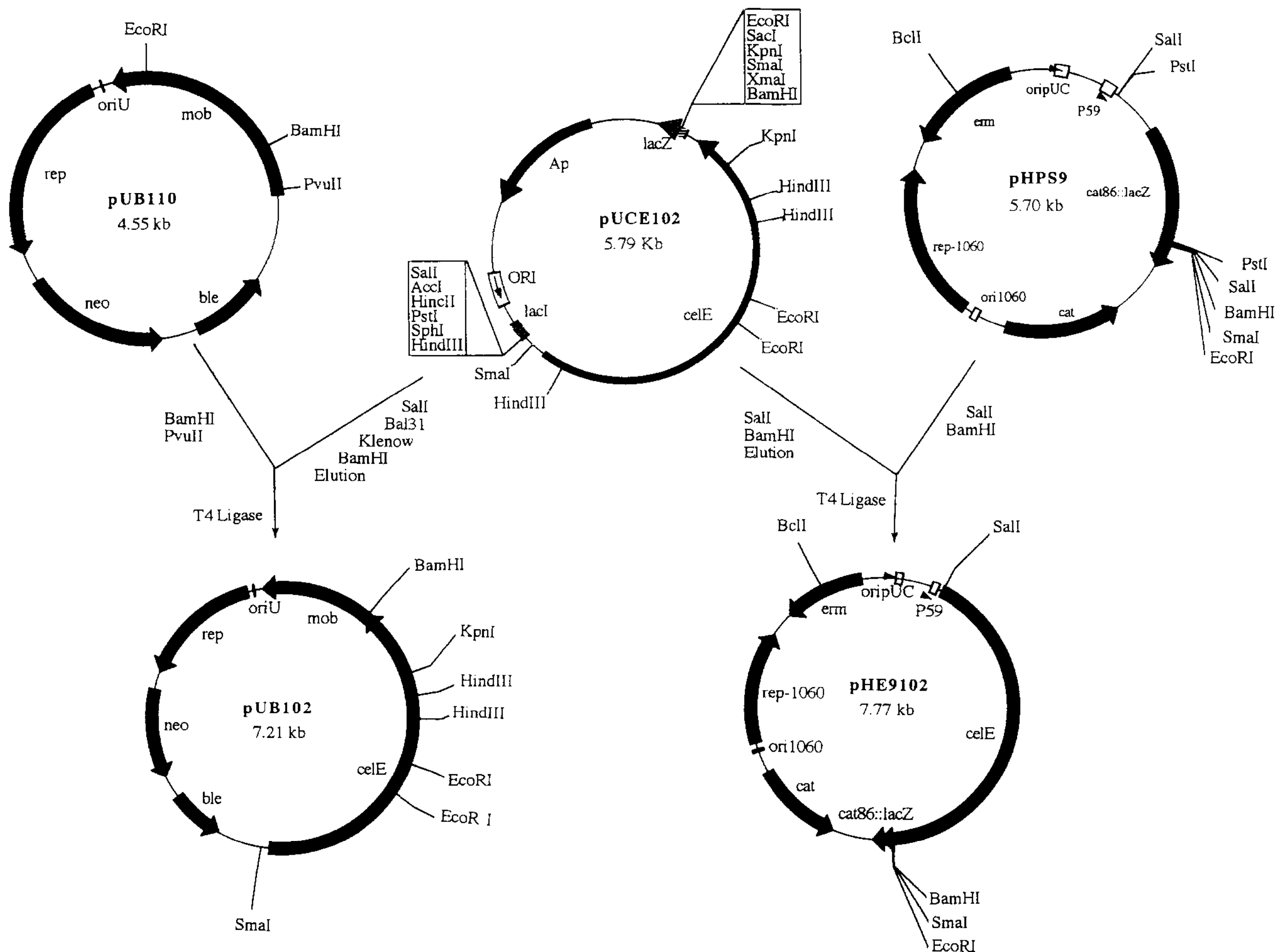


FIG. 1. Overview of the construction of the pUB102 and pHE102 plasmids. Plasmids are not drawn to scale. For details see text.

the original promoter region was found to be intact. Testing of several chosen clones under nonselective conditions revealed their structural and segregational stability (data not shown).

Since the kanamycin resistance marker of pUB110 cannot be used for selection of transformants of thermophilic bacilli due to the thermolability of the encoded kanamycin nucleotidyltransferase (25), a second vector allowing chloramphenicol-resistance selection, pHPS9 (26), was used for subcloning of the *celE* gene. The chloramphenicol acetyltransferase (CAT) gene of pC194 carried by this plasmid has been found to allow the chloramphenicol-resistance selection at growth temperatures up to 68°C (27). In these constructs the regulatory regions of the *celE* gene were left intact and it was cloned in both orientations to the internal vector promoter P59. We did not find any significant differences in the CMCase activity levels between the transformants carrying the gene in the opposite orientations, and only pHE9102 was used further for expression study (Fig. 1).

**Kinetics of EGE production by recombinant *B. subtilis*** *B. subtilis* 168 transformed by plasmid pUB102 was further tested in batch culture for investigating the kinetics of expression of thermostable CMCase activity. It was established that EGE is actively secreted by *B. subtilis* 168 (pUB102) cells into the culture medium (Fig. 2). The level of thermostable CMCase activity in crude cell extract was permanently low during the cultivation period and did not exceed a background level (data not shown). About 60% of the cellulase activity was found is attributed to the stationary phase of growth indicating that transcription of the *celE* gene may be driven by sigma factors expressed during the stationary phase of growth and, probably, specific for the sporulation pathway.

The level of *celE* expression can be significantly increased by a growth temperature shift from 37°C to 42°C (Fig. 2). At this elevated growth temperature the final biomass yield was decreased while the production of thermostable CMCase activity was found increased 2.5-fold as compared with the case of cultivation at 37°C. Thus, the presence of two different factors of stress conditions, the depletion of nutrients at the stationary phase of growth and elevated growth temperature was found to be important for the efficient expression of *celE* in *B. subtilis* 168. It is clear that it is transcribed preferably by other than the vegetative RNA-polymerases of *B. subtilis*. In the 5′-noncoding region of *celE*, two TATAAT sequences homologous to the −10 consensus sequences of the *E. coli* and *B. subtilis* vegetative promoters can be identified 130 bp and 163 bp,

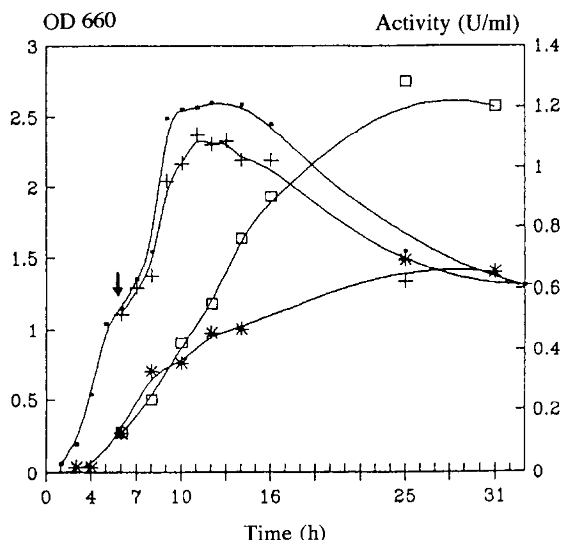


FIG. 2. Growth and thermostable CMCase production by recombinant strain *B. subtilis* 168 (pUB102). The growth curves obtained at 37°C (■), and at 42°C (+), and enzyme activities at 37°C (*) and at 42°C (□) are shown. The start of incubation at 42°C is indicated by an arrow.

respectively, upstream of the start codon (for the sequence see Ref. 28). Homologies with the −35 consensus sequence TTGACA, are, however, less evident and this may serve as indirect evidence that other sigma factors than vegetative recognize the promoter region of the *celE* gene.

**Sporulation events in *B. subtilis* during *celE* expression** When the sporulation-deficient strain of *B. subtilis*, 1A718, was transformed by plasmid pHE9102, thermostable CMCase activity was detected neither in the culture medium nor in the cell extracts (Table 2). At the same time the plasmid isolated from these transformants did not show any structural rearrangements and can be retransformed into strain 168 with the concurrent appearance of the thermostable CMCase activity (data not shown). We observed also that the sporulation efficiency is impeded in the sporulation-proficient strains expressing *celE* (Table 3). These results suggest that the sporulation sigma factor(s) also participate in transcription of this gene in *B. subtilis*. The fact that part of the cell population did not enter the normal sporogenesis pathway after transformation may be explained by a shortage of the sporulation-specific sigma factor(s) under conditions of competition between the host genes and the *celE* gene introduced on the plasmid.

TABLE 2. Thermostable CMCase activity in the culture supernatants of *Bacillus* strains

| Strain, plasmid | CMCase activity (U/ml) |
|---|---|
| *B. subtilis* 168 | 0.10 |
| *B. subtilis* 168 (pUB 102) (growth at 37°C) | 0.65 |
| *B. subtilis* 168 (pUB 102) (growth at 42°C) | 1.22 |
| *B. subtilis* 168 (pHE9102) (growth at 37°C) | 0.51 |
| *B. subtilis* 1A751 | ND[a] |
| *B. subtilis* 1A751 (pHE9102) | 0.67 |
| *B. subtilis* 1A718 (pHPS9R) | 0.07 |
| *B. subtilis* 1A718 (pHPS9R, pHE9102) | 0.06 |
| *B. stearothermophilus* 9A2 | ND |
| *B. stearothermophilus* 9A2 (pHE9102) | ND |
| *B. stearothermophilus* 9A5 | ND |
| *B. stearothermophilus* 9A5 (pHE9102) | ND |

[a] Not detected.

TABLE 3. Effect of *celE* expression on the sporulation efficiency of *B. subtilis* strains

| Strain, plasmid | Viable cell count ($ml^{-1}$) | Spore count ($ml^{-1}$) | Sporulation efficiency (%) |
|---|---|---|---|
| *B. subtilis* 1A751 (pHPS9) | $6.8 \times 10^8$ | $8.0 \times 10^8$ | 100 |
| *B. subtilis* 1A751 (pHE9102) | $5.8 \times 10^8$ | $8.4 \times 10^6$ | 1.5 |
| *B. subtilis* 168 (pHPS9) | $6.1 \times 10^8$ | $3.1 \times 10^8$ | 50.8 |
| *B. subtilis* 168 (pHE9102) | $5.3 \times 10^8$ | $1.2 \times 10^8$ | 22.6 |

**Expressed protein patterns** Earlier we showed that the *celE* gene product is subjected to specific proteolysis in *E. coli* cells (29). The heterologous expression of *celE* in *B. subtilis* is also of no exception. In fact, the protein purified from *B. subtilis* 168 (pHE9102) was degraded to an even greater extent than purified from *E. coli* (Fig. 4). Even when expressed in *B. subtilis* strain 1A 751 which is deficient in two major extracellular proteases (Table 1), it is degraded by its host to the same extent as in strain 168 and gives two very close polypeptide bands with an $M_r$ of around 39,000–40,000 instead of 88,928 coded by the ORF of *celE* (Fig. 4). N-terminal cleavage as predicted by the model of Von Heijne (30), might be expected to occur after the sequence VAA or AAS, conferring to amino acid position 26 or 27, respectively; however, the N-terminal sequences of these two polypeptides were found to be identical and they corresponded to amino acid residues 50 through 59 of the translated sequence (Fig. 3). There are also two putative proteolysis sites on the P-T-rich linker sequence (Fig. 3).

Although all purification buffers included 40 $\mu$M phenylmethylsulfonyl fluoride (PMSF) to inactivate any possible serine protease contaminations during the purification, the possibility of cleavage by proteases with other specificities or structural instability during the purification could not be excluded. Therefore, the culture supernatant of *B. subtilis* 1A751 (pHE9102) in the exponential growth phase was prepared as quickly as possible at 4°C, heated in the sample buffer and directly loaded into the SDS-PAGE for subsequent Western blotting analysis. As can be seen in Fig. 4, these blots showed the same protein patterns with the same molecular weights as the purified proteins. Thus, the protease processing was concluded to occur *in vivo*. This result demonstrates that endoglucanase E is very sensitive even to the minor proteases of *B. subtilis*, and, as a consequence, the enzymatically active protein can be isolated only in the truncated form. In the original host these

TABLE 4. Buffer dependence of ΔEGE capacity factor, *k*, on Superdex column

| Point | Buffer | Capacity factor, *k* |
|---|---|---|
| 1 | 100 mM phosphate-citrate, pH 6.4 | 0.11 |
| 2 | 100 mM phosphate-citrate, pH 6.4 + 0.2 M $Na_2SO_4$ | 0.40 |
| 3 | 50 mM phosphate, pH 7.0 + 0.15 M NaCl | 0.21 |
| 4 | 50 mM phosphate, pH 7.0 + 0.2 M NaCl | 0.33 |
| 5 | 50 mM phosphate, pH 7.0 + 0.2 M $Na_2SO_4$ | 0.34 |
| 6 | 25 mM Tris-HCl, pH 7.5 + 0.05 M NaCl | 0.03 |
| 7 | 25 mM Tris-HCl, pH 7.5 + 0.2 $(NH_4)_2SO_4$ | 0.37 |

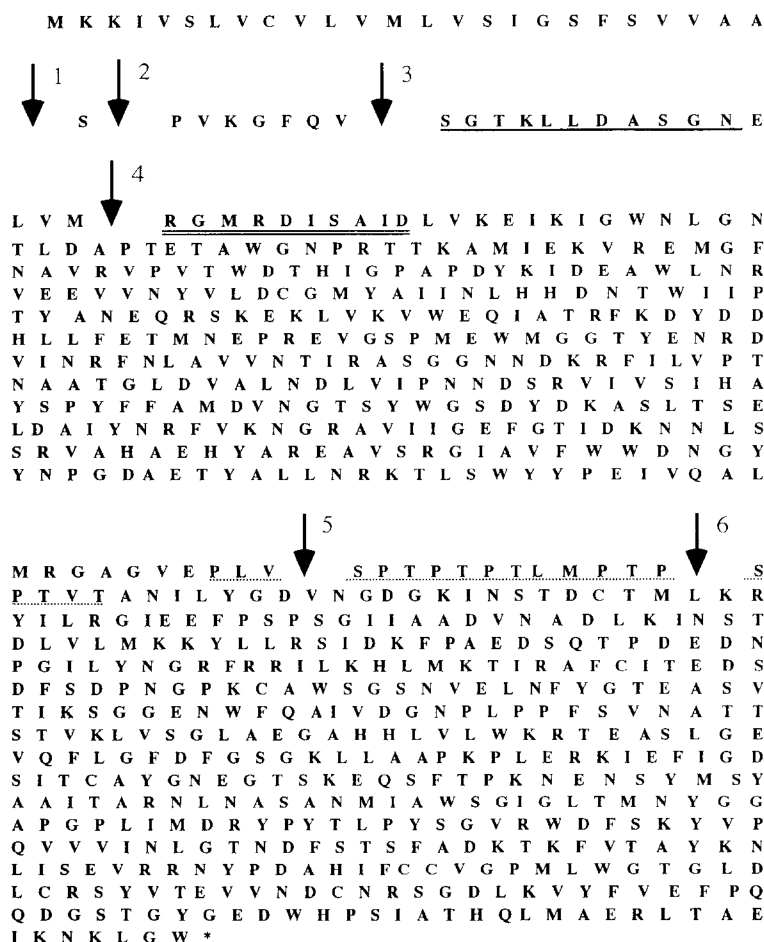

FIG. 3. The translated amino acid sequence of EGE. The proteolysis sites are indicated by arrows: 1 and 2—cleavages as predicted by the model of von Heijne (30); 3 and 4—the actual proteolysis sites in *E. coli* and *B. subtilis*, respectively; 5 and 6—proteolysis sites in the P-T-rich region in both hosts. The N-terminal sequences, determined as described in Materials and Methods, are underlined, by single line—purified from *E. coli*, by double line—purified from *B. subtilis*. Dashed underline indicates the P-T-rich region.

proteolysis sites are probably protected by glycosylation or specific chaperons.

**Transformation of *B. stearothermophilus* strains by plasmid pHE9102** Two *B. stearothermophilus* strains, 9A2 and 9A5, were electrotransformed by plasmid pHE9102. Several transformants were tested for the thermostable CMCase activity, but it could be detected neither in the culture medium nor in the cell extract. Plasmid DNAs from several transformants were reisolated and analyzed either directly or after retransformation into *E. coli*. In all cases the incoming plasmid DNA underwent specific recombinational rearrangements with the chromosome of *B. stearothermophilus* involving the *celE* locus of the plasmid (data not shown).

**Purification of EGE** Since EGE in *B. subtilis* is subjected to proteolysis which removes roughly more than half of its amino acid residues, the remaining truncated domain should exhibit features characteristic to catalytic domains of endoglucanases. Really, as reported in the case of other cellulases (31, 32), we observed retardation of ΔEGE elution from gel-filtration columns. Results, summarized in Table 4, clearly demonstrate that under the wide range of conditions used, with various the ionic strengths and pHs, the retention time of ΔEGE is significantly longer than might be expected from its molecular mass. As we have shown earlier, an ideal SEC for ΔEGE can be obtained only under restricted conditions (31). First, the mobile phase must contain an electrolyte whose concentration and salting-out strength are not high in order to attenuate the electrostatic interactions but not to promote significantly hydrophobic interactions. Second, the mobile-phase pH must be high enough to balance the attractive hydrophobic interactions with the repulsive electrostatic interactions. Nonrandom distribution of hydrophobic amino acid residues in the EGE molecule may explain the hydrophobic nature of the resulting ΔEGE. Indeed, closer examination of Fig. 3 reveals that 50% of the amino acid residues of ΔEGE are hydrophobic.

At the same time this unusual behavior allows single-step purification on the gel-filtration columns, after ammonium sulfate precipitation of the proteins secreted into the culture supernatant. As can be seen in Fig. 5A, ΔEGE is eluted later than it might be expected from its $M_r$, 40 kDa, and even later than cytochrome C (12.3 kDa). On SDS-PAGE of the fractions of this latter peak only the ΔEGE-related bands were detected (Fig. 5B). Besides allowing the purification of this peptide and its derivatives in a single step, these properties may be used also for rapid purification of other proteins by

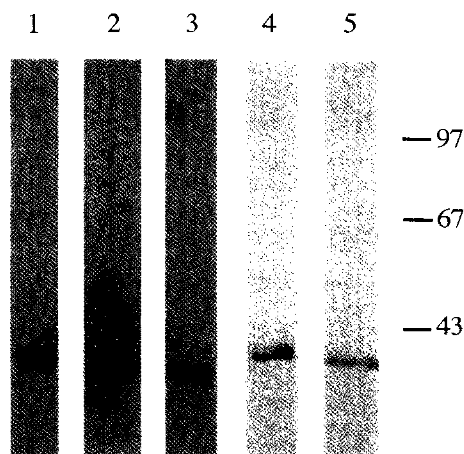


FIG. 4. SDS-PAGE of ΔEGE from the recombinant strains. Lane 1, ΔEGE purified from *E. coli* JM109 (pUCE102); lane 2, ΔEGE purified from *B. subtilis* 168 (pHE9102); lane 3, ΔEGE purified from *B. subtilis* 1A751 (pHE9102); lanes 4 and 5—Western immunoblot using anti-ΔEGE antiserum and total cell extract of *E. coli* JM109 (pUCE102) and the culture supernatant of *B. subtilis* 1A751 (pHE9102), respectively. Positions of molecular mass markers are shown on the extreme right.

fusing them with the ΔEGE domain.

## DISCUSSION

We established here that the *celE* gene from *C. thermocellum* can be expressed in *B. subtilis* utilizing the native promoter sequence and that endoglucanase E is secreted into the culture supernatant of the recombinants. Another endoglucanase gene from the same organism, *celA*, for which much information on its heterologous expression is available, has been successfully expressed under the control of its own promoter in *Lactobacillus plantarum* (5) and *Thermus thermophilus* (8), but its cloning in *B. subtilis* resulted in overexpression effects favoring the survival of the transformants, with rearrangements and deletions of the recombinant plasmids (7). Successful transfer of the *celA* gene to bacilli was achieved by placing the gene under the control of a host's moderate promoter (7). The promoter region of the *celE* gene is probably recognized by other factors than vegetative sigma factors of *B. subtilis* RNA-polymerase. Several facts support this assumption. First, EGE production is mainly attributed to the stationary phase of growth. Second, EGE production can be enhanced under elevated temperature conditions. Finally, expression requires a sporulation-proficient phenotype of the host. Thus, if transcription is driven by the minor sigma factors of *B. subtilis*, expression would be expected to occur at the normal, physiological level, without strong selection against the recombinant plasmid. Localization of the expression bottleneck at the transcription level might also help to explain why no gene dosage effects was observed when *celE* was cloned on the high (pUB110) or low (pHPS9) copy number plasmids. Transcription studies of endoglucanase genes in the homologous host, *C. thermocellum*, revealed that transcripts of *celA*, *celD*, and *celF* could be detected during the late exponential and early stationary growth phases, while the *celC* transcript was found almost exclusively during the early stationary growth phase (33). A mechanism

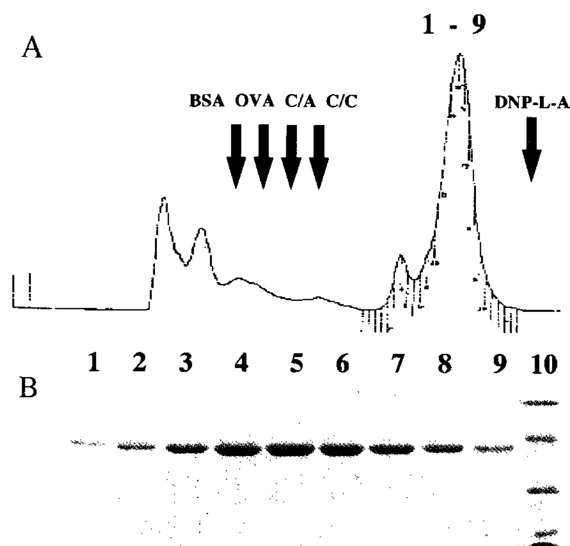


FIG. 5. Chromatogram (A) of the culture supernatant of *B. subtilis* 168 (pHE9102) eluted from Superdex column after ammonium sulfate precipitation, and SDS-PAGE (B) of fractions corresponding to the last peak. (A) Elution volumes of molecular mass standards are shown by arrows: BSA—bovine serum albumin (67 kDa), OVA—ovalbumin (43 kDa), C/A—chymotrypsinogen A (25 kDa), C/C—cytochrome C (12.3 kDa) and DNP-L-Ala—DNP-L-Alanine (255 Da). 1–9—fractions corresponding to the last peak exhibiting endoglucanase activity. Conditions of chromatography as for point 7 (Table 4). (B) Lanes from 1 to 9, fractions 1 to 9 corresponding to the last peak of the chromatogramm (A); lane 10, molecular mass markers, from top to bottom: BSA, ovalbumin, chymotrypsinogen A, cytochrome C.

analogous to catabolite repression, in this case by cellobiose, was proposed to explain the late transcription of these genes (33). In *B. subtilis* cells, however, in the absence of any sugar in the growth medium, occurrence of this type of regulation seems unlikely. Probably, expression of *celE* in *B. subtilis* is regulated by the availability of some minor sigma factors specific to the conditions discussed. This suggestion is supported also by the fact that part of cells expressing *celE* failed to enter the sporulation pathway. This may occur if sporulation-specific sigma factors are branched off to serve the *celE* template leaving the housekeeping functions on sporulation. It is doubtful that EGE directly affects sporulation events, since it is localized extracellularly and there is no evident substrate target for it.

For most microbial β-1,4-glucanases studied to date, the presence of distinct domain structures, composed of catalytic domain(s) and a cellulose binding domain connected by short linker sequence(s) has been demonstrated by deletion and proteolysis analyses (for review see Ref. 34). The linker sequence, rich in proline and/or hydroxyamino acid residues, is very sensitive to proteolytic attack. Its removal may mask the protease-sensitive sites between the domains (35). Clostridial enzymes, however, are organized into the high-molecular-weight macromolecular complex called the cellulosome, and linker sequences connect the catalytic domain and the highly conserved duplicated region responsible for attachment to a cellulase integrating protein, CipA (36). During heterologous expression, cellulases are "naked" and, therefore, the linker sequences are hydrolyzed, as we observed during *celE* expression in *E. coli* or *B. subtilis*.

Moreover, some degree of N-terminal proteolysis downstream of the putative signal peptidase recognition site was also observed. This, on the other hand, is not always the case. The *celA* gene product, for example, appeared to be resistant to hydrolysis in the course of expression in *B. subtilis* (7).

Since the amino acid distribution in ΔEGE is not random, with 50% hydrophobic residues, its behavior on gel-filtration columns is determined by other factors in addition to molecular mass. As we demonstrated earlier, the main cause of adsorption of cellulolytic enzymes on polysaccharide matrices is hydrophobic interaction (31) and the same interaction is concluded to play a major role in the observed retardation of ΔEGE elution from the gel-filtration column.

Since no any data on cellulolytic activity in *B. stearothermophilus* strains are available, it is difficult to speculate at which site on the chromosome the recombination exchange involving the *celE* locus occurs. Probably, local homology including an AT-rich region, a common feature of strongly expressed promoter regions in *Bacillus*, is mainly responsible for the observed recombinational event.

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## REFERENCES


1. **Sacco, M., Millet, J., and Aubert, J.-P.:** Cloning and expression in *Saccharomyces cerevisiae* of a cellulase gene from *Clostridium thermocellum*. Ann. Microbiol. (Inst. Pasteur), **135A**, 485–489 (1984).
2. **Silva, A., Benitez, J., and Hollenberg, C. P.:** Endoglucanase A gene fusion vectors for monitoring protein secretion and glycosylation in yeast. Anal. Biochem., **197**, 290–295 (1991).
3. **Wong, W. K. R., Curry, C., Parekh, R. S., Parekh, S. R., Wayman, M. D., Davies, R. W., Kilburn, D. G., and Skipper, N.:** Wood hydrolysis by *Cellulomonas fimi* endoglucanase and exoglucanase coexpressed as secreted enzymes in *Saccharomyces cerevisiae*. Bio/Technology, **6**, 713–719 (1988).
4. **Bates, E. E. M., Gilbert, H. J., Hazlewood, G. P., Huckle, G., Laurie, J. I., and Mann, S. P.:** Expression of a *Clostridium thermocellum* endoglucanase gene in *Lactobacillus plantarum*. Appl. Environ. Microbiol., **55**, 2095–2097 (1989).
5. **Scheirlinck, T., Mahillon, J., Joos, H., Dhaese, P., and Michiels, F.:** Integration and expression of α-amylase and endoglucanase genes in the *Lactobacillus plantarum* chromosome. Appl. Environ. Microbiol., **55**, 2130–2137 (1989).
6. **Baik, B. H. and Pack, M. Y.:** Expression of *Bacillus subtilis* endoglucanase gene in *Lactobacillus acidophilii*. Biotechnol. Lett., **12**, 919–924 (1990).
7. **Soutschek-Bauer, E. and Staudenbauer, W. L.:** Synthesis and secretion of a heat-stable carboxymethylcellulase from *Clostridium thermocellum* in *Bacillus subtilis* and *Bacillus stearothermophilus*. Mol. Gen. Genet., **208**, 537–541 (1987).
8. **Lasa, I., de Grado, M., de Pedro, M. A., and Berenguer, J.:** Development of *Thermus-Escherichia* shuttle vectors and their use for expression of the *Clostridium thermocellum celA* gene in *Thermus thermophilus*. J. Bacteriol., **174**, 6424–6431 (1992).
9. **Brestic-Goachet, N., Gunasekaran, P., Gami, B., and Baratti, J.-C.:** Transfer and expression of an *Erwinia chrysanthemi* cellulase gene in *Zymomonas mobilis*. J. Gen. Microbiol., **135**, 893–902 (1989).
10. **Wood, B. E. and Ingram, L. O.:** Ethanol production from cellobiose, amorphous cellulose, and crystalline cellulose by recombinant *Klebsiella oxytoka* containing chromosomally integrated *Zymomonas mobilis* genes for ethanol production and plasmids expressing thermostable cellulase genes from *Clostridium thermocellum*. Appl. Environ. Microbiol., **58**, 2103–2110 (1992).
11. **Joliff, G., Edelman, A., Klier, A., and Rapoport, G.:** Inducible secretion of a cellulase from *Clostridium thermocellum* in *Bacillus subtilis*. Appl. Environ. Microbiol., **55**, 2739–2744 (1989).
12. **Petit, M. A., Joliff, G., Mesas, J. M., Klier, A., Rapoport, G., and Erlich, S. D.:** Hypersecretion of a cellulase from *Clostridium thermocellum* in *Bacillus subtilis* by induction of chromosomal DNA amplification. Biotechnology (NY), **8**, 559–563 (1990).
13. **Hall, J., Hazlewood, G. P., Surani, M. A., Hirst, B. H., and Gilbert, H. J.:** Eucaryotic and procaryotic signal peptides direct secretion of a bacterial endoglucanase by mammalian cells. J. Biol. Chem., **265**, 19996–19999 (1990).
14. **Hall, J., Ali, S., Surani, M. A., Hazlewood, G. P., Clark, A. J., Simons, J. P., Hirst, B. H., and Gilbert, H. J.:** Manipulation of the repertoire of digestive enzymes secreted into the gastrointestinal tract of transgenic mice. Bio/Technology, **11**, 376–379 (1993).
15. **Stackebrandt, E. and Teuber, M.:** Molecular taxonomy and phylogenetic position of lactic acid bacteria. Biochimie, **70**, 317–324 (1988).
16. **Dean, D. H. and Zeigler, D. R.:** Bacillus genetic stock center catalog. Fourth edition. The Ohio State University, Columbus (1989).
17. **Kusaoke, H., Hayashi, Y., Kadowaki, Y., and Kimoto, H.:** Optimum conditions for electric pulse-mediated gene transfer to *Bacillus subtilis* cells. Agric. Biol. Chem., **53**, 2441–2446 (1989).
18. **Sambrook, J., Fritsch, E. F., and Maniatis, T.:** Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989).
19. **O'Sullivan, K. J. and Klaenhammer, T. R.:** Rapid mini-prep isolation of high-quality plasmid DNA from *Lactococcus* and *Lactobacillus* spp. Appl. Environ. Microbiol., **59**, 2730–2733 (1993).
20. **Aminov, R. I., Gribanova, L. K., Golovchenko, N. P., Kataeva, I. A., Tsoi, T. V., and Akimenko, V. K.:** Cloning and expression of a *Clostridium thermocellum* endoglucanase gene in Gram-negative bacteria. Genetika, **26**, 1391–1398 (1990).
21. **Laemmli, U. U.:** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**, 680–685 (1970).
22. **Schwarz, W. H., Bronnenmeier, K., Grabnitz, F., and Staudenbauer, W. L.:** Activity staining of cellulases in polyacrylamide gels containing mixed linkage β-glucans. Anal. Biochem., **164**, 72–77 (1987).
23. **Tsoi, T. V., Bukhtjarova, M. G., Aminov, R. I., Golovchenko, N. P., Kataeva, I. A., Kosheleva, I. A., Akimenko, V. K., and Boronin, A. M.:** Cloning and expression of *Clostridium thermocellum cel* genes in *E. coli* and *B. subtilis*. Genetika, **26**, 1349–1358 (1990).
24. **Zyprian, E. and Matzura, H.:** Characterization of signals promoting gene expression on the *Staphylococcus aureus* plasmid pUB110 and development of a Gram-positive expression vector system. DNA, **5**, 219–225 (1986).
25. **Matsumara, M., Katakura, Y., Imanaka, T., and Aiba, S.:** Enzymatic and nucleotide sequence studies of a kanamycin-inactivating enzyme encoded by a plasmid from thermophilic bacilli. J. Bacteriol., **160**, 413–420 (1984).
26. **Haima, P., van Sinderen, D., Schotting, H., Bron, S., and Venema, G.:** Development of a β-galactosidase α-complementation system for molecular cloning in *Bacillus subtilis*. Gene, **86**, 63–69 (1990).
27. **Soutschek-Bauer, E., Scholz, W., Grill, E., and Staudenbauer, W. L.:** Thermostability and superhelicity of plasmid DNA in *Bacillus stearothermophilus*. Mol. Gen. Genet., **209**, 575–579 (1987).
28. **Hall, J., Hazlewood, G. P., Barker, P. J., and Gilbert, H. J.:** Conserved reiterated domains in *Clostridium thermocellum* endoglucanase are not essential for catalytic activity. Gene, **69**,

29–38 (1988).
29. **Aminov, R. I., Singh, R. N., Golovchenko, N. P., and Akimenko, V. K.:** Specific proteolysis of an endoglucanase of *Clostridium thermocellum* during heterologous expression in *Escherichia coli*. Genetika, **29**, 217–224 (1993).
30. **von Heijne, G.:** A new method for predicting signal sequence cleavage sites. Nucleic Acids Res., **14**, 4683–4690 (1986).
31. **Golovchenko, N. P., Kataeva, I. A., and Akimenko, V. K.:** Elucidation of the role of hydrophobic interactions in the adsorption of endo-1,4-β-glucanases on polysaccharides. Enzyme Microb. Technol., **14**, 327–331 (1992).
32. **Shoseyov, O. and Doi, R. H.:** Essential 170-kDa subunit for degradation of crystalline cellulose by *Clostridium cellulovorans* cellulase. Proc. Natl. Acad. Sci. USA, **87**, 2192–2195 (1990).
33. **Mishra, S., Beguin, P., and Aubert, J.-P.:** Transcription of *Clostridium thermocellum* endoglucanase genes *celF* and *celD*. J. Bacteriol., **173**, 80–85 (1991).
34. **Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C., and Warren, R. A. J.:** Domains in microbial β-1,4-glucanases: sequence conservation, function, and enzyme families. Microbiol. Rev., **55**, 303–315 (1991).
35. **Shen, H., Schmuck, M., Pilz, I., Gilkes, N. R., Kilburn, D. G., Miller, R. C., and Warren, R. A.:** Deletion of the linker connecting the catalytic and cellulose-binding domains of endoglucanase A (*CenA*) of *Cellulomonas fimi* alters its conformation and catalytic activity. J. Biol. Chem., **266**, 11335–11340 (1991).
36. **Beguin, P. and Aubert, J.-P.:** The biological degradation of cellulose. FEMS Microbiol. Rev., **13**, 25–58 (1994).