Inducible Secretion of a Cellulase from *Clostridium thermocellum* in *Bacillus subtilis*

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A host-vector system for inducible secretion during the logarithmic growth phase in *Bacillus subtilis* has been developed. The *B. subtilis* levansucrase gene promoter and the region encoding its signal sequence have been used. The endoglucanase A of *Clostridium thermocellum* was used as a model protein to test the efficiency of the system. Effective inducible secretion of the endoglucanase A was observed when either the levansucrase signal sequence or its own signal sequence was used. Expression of the endoglucanase A in different genetic backgrounds of *B. subtilis* showed that its regulation was similar to that of levansucrase, and high enzyme activity was recovered from the culture supernatant of a hyperproducing *B. subtilis sacU(Hy)* strain. The molecular weight of 46,000 estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the secreted endoglucanase A is compatible with the calculated molecular weight of the mature polypeptide.

Various heterologous proteins have been secreted in *Bacillus subtilis*, and most of the secretion vectors developed use the α-amylase secretory system (27, 28, 30), although, more recently, protease export systems have been used (13, 14, 28, 35, 37). However, expression from these systems occurs during stationary phase, when most of the secreted enzymes of *B. subtilis*, including the major proteases, are secreted. Here, we describe a secretion vector based on the *B. subtilis* levansucrase (Lvs) system, which is active during the logarithmic growth phase. Lvs is encoded by the sacB gene and expressed from a strong sucrose-inducible promoter located in the closely linked sacr locus. The control of its expression has been studied extensively (12, 33), and several regulatory up mutants are available: sacQ(Hy), sacS(Hy), and sacU(Hy). The sacQ(Hy), sacS(Hy), and sacU(Hy) mutants produce high levels of Lvs (19, 20). The sacQ, sacS, and sacU gene products are transcriptional regulators of the expression of sacB. The sacQ and sacU genes act on a region located near or just upstream from the promoter, and the sacS gene, which is involved in the induction process, acts on the palindromic structure located between the promoter and the ribosome binding site (3, 16). These factors make it a suitable candidate for the construction of secretion systems for heterologous proteins. This system has already been used to overproduce an intracellular protein, the cathecol 2,3-dioxygenase, encoded by the xylE gene of *Pseudomonas putida* (38). Fusions of the Lvs gene of *B. subtilis* and the α-amylase gene of *Bacillus licheniformis* containing various lengths of the mature parts of both proteins have also been described (18). Furthermore, we recently reported the secretion of the TEM β-lactamase of *Escherichia coli* using the sacB system (11).

The extracellular endoglucanase A (EGA; 1,4-β-d-glucan gluconohydrolase [EC 3.2.1.4]) of the thermophilic anaerobic bacterium *Clostridium thermocellum* encoded by the celA gene has been used as a model protein to test the efficiency of the sacB secretion system in *B. subtilis*. This protein was chosen for its convenient size (22), its successful expression in various microorganisms (10, 25, 32), and its enzymatic activity, which is easily detectable directly on plates (10). Furthermore, EGA has been characterized and purified (22, 31), and the DNA sequence (5) of its gene and the sites of transcription initiation (7) are known. The calculated molecular weight of mature EGA is 49,125, but on sodium dodecyl sulfate-polyacrylamide gels of *C. thermocellum* culture supernatants, it appears as a 56,000-molecular-weight band believed to be due to glycosylation (5). These factors facilitate the use of this protein as a model for heterologous secretion by *B. subtilis*.

Here, we show that *B. subtilis* can secrete enzymatically active EGA into the culture medium after transformation with hybrid plasmids containing precise fusions, after induction by sucrose. We also compare the efficiency of the Lvs and EGA signal sequences when fused to the structural gene of EGA.

**MATERIALS AND METHODS**

Strains, bacteriophage, plasmids, and media. The strains and plasmids used are listed in Table 1. The phage M3mp18-amy was used for the oligonucleotide site-directed mutagenesis. For growth of *E. coli*, Luria broth (21) was used and supplemented with ampicillin (100 μg/ml), chloramphenicol (10 μg/ml), kanamycin (50 μg/ml), or tetracycline (10 μg/ml). For growth of *B. subtilis*, sporulation broth (29) was used and supplemented with chloramphenicol (5 μg/ml) or kanamycin (5 μg/ml) for selection and 2% (wt/vol) sucrose for induction of the sacB promoter. *E. coli* was transformed by the method of Cohen et al. (9), and *B. subtilis* was transformed by the method of Anagnostopoulos and Spizizen (2).

Enzyme assays. Carboxymethyl cellulose (CMC) (no. C-4888, medium viscosity; Sigma Chemical Co.) was used as the substrate for the EGA. Endoglucanase activity was detected by the Congo red assay on plates by the method of Cornet et al. (10). All enzyme assays were performed in PC buffer (50 mM K2HPO4, 12 mM citric acid, pH 6.3) at 60°C. Carboxymethyl cellulase (CMC-ase) activity was measured by the method of Béguin et al. (6). One unit of activity was defined as the amount of enzyme that released 1 μmol of glucose equivalent per min. Cellulase activity was detected in polyacrylamide gels by using Congo red-stained agar replicas by the method of Béguin (4).
Oligonucleotide site-directed mutagenesis. The procedure used for oligonucleotide site-directed mutagenesis was essentially that described by Carter et al. (8). pGJ3 (see Fig. 4) was used to produce a single-stranded template which was primed with both the mutagenic primer DGF3 (5’ AAAAA GCACACCTGCGGCAAACGCTTGAGT) and a selection primer, SEL1 (5’ AAGGAATCTGCGTGCCATCAC 3’) to remove the selectable marker from the minus strand and was transfected into HB2154 cells, which are repair deficient and select against the marker; therefore, a selection for progeny phage derived from replication of the minus strand was obtained. The 15 5’-nucleotides of the mutagenic primer DGF3 are complementary to the first 15 nucleotides of the EGA mature sequence, and the 15 3’-nucleotides are complementary to the last 15 nucleotides of the Lvs signal sequence contained in pAE101 (11). Southern blotting was performed by the method of Carter et al. (8) after a wash in 6x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature and at 45°C, 55°C, and 65°C, successively; the mutants containing the in-frame fusion between the Lvs signal sequence and the EGA mature sequence hybridized strongly to DGF3 after the last wash. The mutation corresponding to a deletion of 19 nucleotides of the EGA signal sequence was verified by dideoxy sequencing (26) by using the synthetic oligoprimer DGF4 (5’ TCGGCAATAGAAGTAGTGG 3’), which hybridizes downstream of the deleted region.

Construction of pGJ1, pGJ2, and pAE119. Plasmid pGJ1 was constructed by a three-way ligation of (i) 50 ng of the vector pUC8 cut with BamHI and HindIII, (ii) 25 ng of the 440-base-pair (bp) BamHI-Rsal fragment of the sacR region containing the sacB promoter and its regulation region from pBS430 (3, 16), and (iii) 100 ng of the 2.2-kbp Draf-HindIII fragment of the plasmid pCT105 (10) containing the putative ribosome binding site, the region encoding EGA (signal sequence and mature sequence) (5), and the putative termination site of the gene (7) (Fig. 1). The 440-bp and 2.2-kbp fragments were isolated from a 1.5% agarose gel and were purified by electroelution from the agarose gel. The desired construct, pGJ1, was isolated as an Ap' transformant of E. coli exhibiting CMC-ase activity (CMC' phenotype) in the Congo red plate assay (10). To test the secretion of EGA in B. subtilis, pC194 (15), a plasmid of 2.91 kbp containing a B. subtilis functional origin of replication and an antibiotic resistance marker (chloramphenicol), was ligated to pGJ1 at the HindIII site, resulting in plasmid pGJ2.

The plasmid pAE119 was used for the deletion of the sacRB region in B. subtilis, by recombination. pBS431 (1) contains the sacRB region on contiguous HindIII fragments. The plasmid was linearized with ClaI, which cuts in the sacB gene, and was treated with BAL 31 exonuclease to delete the entire sacRB region. After filling of ragged ends, the fragment was ligated to the aphA3 gene from Streptococcus faecalis to result in pAE119, which is not replicative in B. subtilis. The aphA3 gene used is included in a 1.5-kbp ClaI fragment.

![FIG. 1. Construction of PGJ1 (see Materials and Methods for details). Solid bars represent DNA fragments containing the sacB region or the celA gene. Open bars represent regions from pCT105 flanking the celA gene. Arrows indicate the direction of transcription of the genes.](http://aem.asm.org/DownloadedFrom)
VOL. 55, 1989

FIG. 2. Expression of celA gene from plasmid pGJ2 in different B. subtilis strains after induction by sucrose. ●, Bacterial growth of the wild-type strain. The mutants strains gave the same growth curve. The symbols correspond to CMC-ase activity per milliliter of supernatant where the host strain was: ○, sacU(Hy); △, sacS(Hy); □, sacS(Con); ■, wild type. ○, CMC-ase activity per milliliter of supernatant in a noninduced sacU(Hy) strain.

fragment from pAT21 (34). After transformation of B. subtilis with pAE119, recombinants were KmT and LysT, suggesting a deletion in the sacRB region by a double-crossover event.

RESULTS

Expression of the celA gene in B. subtilis under control of the sacR region. A construct for the expression of EGA was made by a three-way ligation, as mentioned in Materials and Methods. The resulting plasmid, pGJ2, was introduced into different B. subtilis strains: 168 (wild type), sacS(Con), sacS(Hy), and sacU(Hy) (Table 1). CmT transformants of B. subtilis carrying the C. thermocellum celA gene on the multicopy plasmid pC194 were identified on the basis of enlarged zones of CMC hydrolysis when the bacteria were induced by sucrose. However, the halo size varied, depending on the host strain. Endoglucanase assays of liquid culture supernatants demonstrated substantial sucrose-inducible secretion of EGA (Fig. 2) (Table 2). These results suggested that the regulation of the expression of the celA gene was the same as that of the sacB gene. No expression of the celA gene was detected in the absence of sucrose in the inducible strains. The highest level of EGA secretion was obtained with the sacU(Hy) mutant, which also hypersecreted levansucrase, producing about 100-fold more of it than wild-type cells produced. EGA secretion by this strain was only about eightfold higher than that by the induced transformed wild type, even though the gene was expressed from a multicopy plasmid. However, the expression level in a sacU(Hy) strain of sacB on a multicopy plasmid is only 5- to 10-fold higher than the level obtained in a sacU+ strain (unpublished results). This may indicate limitation factors in the expression or secretion mechanisms. The expression of celA in a sacS(Con) mutant was hyperinducible, as it has already been observed with the expression of sacB in the same background (J.-A. Lepesant, Ph.D. thesis, Université Paris VII, 1974). B. subtilis possesses an endogenous β-1,4-endoglucanase activity (24). However, in strain B. subtilis sacU(Hy) (pGJ2), which presented a higher endogenous activity than the wild-type strain, this accounts for only about 2% of CMC-ase activity.

Endoglucanase assays showed that cell extracts of strain sacU(Hy) (pGJ2) contained about 20% of the total EGA activity, indicating that most of the EGA (80%) was secreted in the medium (data not shown).

Secretion of EGA by the levansucrase signal sequence and regulation region sacR. The DNA region encoding the signal sequence of levansucrase and carrying sacR was fused to the region encoding mature EGA by oligonucleotide site-directed mutagenesis in an M13 vector (8) (Fig. 3). The vector M13mp18-am4 (8), which has an amber mutation in an essential phage gene, was cut with BamHI and HindIII and

<table>
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<tr>
<th>Strain</th>
<th>Relevant phenotype</th>
<th>Plasmid</th>
<th>Induction by sucrose</th>
<th>CMC-ase activity (U/ml)</th>
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<tr>
<td>168</td>
<td>Wild type</td>
<td>pC194</td>
<td>+</td>
<td>&lt;0.01</td>
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<td></td>
<td></td>
<td>pGJ2</td>
<td>-</td>
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<td>+</td>
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<tr>
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<td>SacS(Con)</td>
<td>pC194</td>
<td>+</td>
<td>&lt;0.01</td>
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<td></td>
<td></td>
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<td>-</td>
<td>0.11</td>
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<tr>
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<td></td>
<td>pGJ2</td>
<td>+</td>
<td>0.80</td>
</tr>
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* The activity was measured in culture supernatants recovered at late log phase.

FIG. 3. Fusion of the region encoding the mature part of EGA to the region encoding the signal sequence of Lvs by site-directed mutagenesis. DGF3 is the mutagenic primer. The sequence deleted from pGJ3 (Fig. 4) is shown as a hairpin.
ligated to (i) a BamHI-NaeI fragment of 0.55 kbp from the plasmid pAE101 (11) carrying sacR and the levansucrase signal sequence, and to (ii) a NaeI-HindIII fragment of 2.14 kbp from the plasmid pCT105 (10), containing the DNA encoding the mature EGA and 19 nucleotides from the signal sequence of EGA (Fig. 4). The resulting plasmid was called pGJ3. The 19 nucleotides belonging to the EGA signal sequence were deleted from pGJ3, and constructs containing the desired sequence were named pGJ4 (Fig. 4). However, the plasmids contained an insertion of only about 0.9 kbp in M13mp18, instead of the expected 2.7 kbp, corresponding to a deletion of about 1.8 kbp in the 3’ region of the insert. This was verified by sequencing with the universal primer. The complete gene fusion was reconstructed. Three fragments, (i) the 0.58-kbp fragment BamHI-AvaII from pGJ4, containing the in-frame fusion between the signal sequence of Lvs and the mature sequence of EGA; (ii) the 2.09-kbp partial AvaII-HindIII fragment from pCT105 (Fig. 1); and (iii) the vector pUC8, cut with BamHI-HindIII, were ligated together. The resulting plasmid, containing the sacB-celA translational fusion, was named pGJ5 (Fig. 4).

To test the secretion in B. subtilis of EGA resulting from this fusion, the plasmid pC194 was ligated at the HindIII site of pGJ5. The resulting plasmid, pGJ6, was introduced into B. subtilis sacU(Hy) (QB136). All of the Cm’ transformants tested possessed a sucrose-inducible CMC- phenotype on the basis of the Congo red plate assay. The supernatant of one clone was assayed for its activity on CMC after induction by sucrose, and the level of activity in the supernatant was the same as that of QB136(pGJ2). This result seemed to indicate that the signal sequences of Lvs and EGA are equally efficient for secretion in B. subtilis.

Molecular weight and stability of the EGA secreted by B. subtilis. Analysis of the supernatant of sucrose-induced B. subtilis QB136 harboring pGJ6 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a protein band with an estimated molecular weight of 46,000 which was not detected in the controls (Fig. 5). The same profile was obtained for QB136(pGJ2). This band was shown to possess CMC-ase activity by replication onto agarose gels containing CMC (data not shown). The molecular weight of this band is compatible with the calculated molecular weight of mature EGA, 49,125, as deduced from the nucleotide sequence (5). Even in a sacU(Hy) mutant, which is known to overproduce proteases (17), no loss of CMC-ase activity was observed during an overnight incubation of cells grown to stationary phase, indicating that EGA was resistant to proteolytic degradation. These observations indicate that EGA is secreted into the culture medium in a complete and stable form. Strains QB136 and QB136(pGJ6) were induced with sucrose, and supernatants were assayed for Lvs activity. The untransformed strain produced three times more Lvs activity than did the strain carrying pGJ6. This result was in agreement with densitometer tracings of Coomassie blue-stained polyacrylamide gels (Fig. 5) and suggests competition between the sacB gene and the heterologous sacB/celA gene for expression or secretion (or both).

Expression of the sacB-celA fusion in a sacRB-deleted background. To test whether sacB expression from the chromosomal gene interferes with celA over-expression, the sacRB region was deleted from the chromosome of host strains. To achieve this, host strains were transformed with pAE119 (see Materials and Methods) and Km’ transformants were selected. In more than 98% of transformants, the sacRB region was replaced by aphA3, as shown by the absence of Lvs activity (data not presented). Plasmid pGJ6 was intro-
VOL. 55, 1989

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Aliquots (25 μl) of the supernatants from B. subtilis QB136 late-log-phase cultures, concentrated 25 times on a Amicon membrane, were applied to a 10% polyacrylamide gel. The figure shows densitometer tracings of Coomassie blue-stained gels: (a) QB136; (b) QB136(pGJ6); (c) molecular size reference markers: phosphorylase b (94 kilodaltons [kDa]), bovine serum albumin (67 kDa), levanu-crase (50 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa).

duced into B. subtilis QB671 in which the sacRB region had been deleted as described above. QB671 is isogenic with QB907 (Lepesant, thesis), except that it is sacA (sucrose inhibits growth in sacA sacB strains; data not shown). Strains QB671(pGJ6) and QB671ΔsacRB(pGJ6) were grown in broth and induced with sucrose. The CMC-ase activities of the supernatants were assayed and were indistinguishable from each other.

DISCUSSION

The heterologous protein EGA of C. thermocellum was expressed and secreted from B. subtilis by using the sacB promoter after induction by sucrose. EGA is secreted during the logarithmic growth phase and accumulates in the medium. The concentration of EGA is highest during the stationary phase, suggesting that the protein is secreted in a stable form. Efficient secretion was obtained by using either the signal sequence of Lvs or its own signal sequence. Analysis of these two signal sequences showed no significant differences from typical signal sequences from gram-positive bacteria. Expression of the enzyme in different genetic backgrounds of B. subtilis (sacS(Con), sacS(Hy)), and sacU(Hy)] showed that its regulation was as that of the sacB gene, and high enzyme activity was recovered from the culture fluid of a sacU(Hy) strain of B. subtilis. The size of the protein (~46 kilodaltons) is compatible with the molecular weight deduced from the sequence of the mature protein.

The level of Lvs secretion decreases when the celA gene is expressed from the sacB promoter on the multicopy vector pCT194, suggesting competition between the homologous and heterologous system in expression or secretion (or both). However, in a ΔsacRB strain the level of secreted EGA is not increased. Presumably, therefore, the presence of a single copy of the sacB gene on the chromosome does not compete significantly with the expression of genes from the sacB promoter on a multicopy plasmid.

The EGA activity in the supernatant of a sacU(Hy) strain was 0.8 U/ml. Because the specific activity of purified EGA is 140 U/mg (23), this corresponds to a protein concentration of approximately 1 mg/liter per OD660 unit. This value compares favorably with those obtained for EGA production in C. thermocellum and E. coli (10).

Analyses of the limiting factors of the system are in progress, with a view to improve the expression of heterologous genes. Secretion of an eucaryotic gene will also be tested in order to assess possible biotechnological applications of this system.

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