Genes encoding xylan and β-glucan hydrolysing enzymes in *Bacillus subtilis*: characterization, mapping and construction of strains deficient in lichenase, cellulase and xylanase

Monika Wolf, Attila Geczi, Ortwin Simon and Rainer Borriss

The gene encoding extracellular xylanase (xynA) was amplified as a 770 bp DNA fragment from *Bacillus subtilis* 168 chromosomal DNA by PCR. The genes encoding endo-β-1,4-glucanase (eglS) and endo-β-1,3-1,4-glucanase (bglS) were isolated from a genomic library of *B. subtilis* 168. The sequences of xynA and eglS were identical to those of the xylanase and cellulase genes from *B. subtilis* PAP115. Integrative plasmids containing DNA fragments with deletions in the coding region of the genes were constructed and used to replace the chromosomal eglS, bglS and xynA genes of *B. subtilis* 168. Strains without any detectable activity against xylan (Xyn-), carboxymethylcellulose (Egl-) or mixed linked β-1,4-glucan (Egl- Bgl-) were obtained. The genes were mapped at 170° (eglS), 175° (xynA) and 340° (bglS) on the *B. subtilis* chromosome.

Keywords: *Bacillus subtilis*, genome sequencing, gene mapping, xylanase, cellulase, lichenase

INTRODUCTION

Cellulose (insoluble fibres of β-1,4-glucan) and hemicellulose (non-cellulosic polysaccharides including glucans, mannans and xylans) are the major constituents of plant cell walls. β-1,4-Xylans are mainly found in secondary cell walls of plants, the major component of woody tissue (Thomson, 1993). The mixed linked 1,3-1,4-β-glucans form the major part of cell walls of cereals like oat and barley. The aerobic soil bacterium *Bacillus subtilis* secretes different hydrolases enabling this organism to use external cellulotic and hemicellulosic substrates. Three types of β-glucan endohydrolase able to degrade 1,3-1,4-β-glucans but distinguished by fine differences in substrate specificity are known. Two of them, endo-1,4-β-glucanase or carboxymethylcellulase (CMCase, cellulase, EC 3.2.1.4) and endo-1,3-1,4-β-glucanase or lichenase (EC 3.2.1.73) are secreted by *B. subtilis*. The third β-glucan endohydrolase (endo-1,3(4)-β-glucanase, EC 3.2.1.6) able to hydrolyse laminarin and barley glucan was detected only in *Rhizopus arrhizus* (Anderson & Stone, 1975). Lichenase encoded by the bglS gene is restricted in its substrate range to mixed linked β-glucans. Only 1,4-linkages adjacent to 1,3-linkages are hydrolysed. CMCase encoded by the eglS gene cleaves carboxymethylcellulose (CM-cellulose) and 1,3-1,4-β-glucans by hydrolysing internal 1,4-β-linkages next to 1,4-linked glucose residues (Anderson & Stone, 1975). Endo-1,4-β-xylanase (xylanase, 1,4-β-xylan xylanohydrolase, EC 3.2.1.8), encoded by the xynA gene, is a component of the complex spectrum of different activities produced by *B. subtilis* to break down plant cell walls (Takahashi & Hashimoto, 1963).

The bglS and eglS genes have been isolated from industrial *B. subtilis* strains and the amino acid sequences of their products have been deduced (Murphy et al., 1984; MacKay et al., 1986). Despite related substrate specificity, the two gene products do not show any similarity at the level of primary sequence and they belong to different families of β-glucan hydrolases. The structure of the xynA gene cloned from *B. subtilis* PAP115 has also been reported. Amino acid sequences deduced from the coding region of the xynA genes isolated from *B. subtilis* and *B. polymyxa* share more than 50% identity (Paice et al., 1986). Unfortunately, the eglS, bglS and xynA genes of *B. subtilis* 168 have not been structurally characterized.

The bglS gene has been mapped by PBS1 transduction in

**Abbreviations**: AZCL, Azurine-Crosslinked; CMCase, carboxymethylcellulase; MUG, 4-methylumbelliferyl-β-D-galactopyranoside.

The EMBL accession numbers for the nucleotide sequences reported in this paper are Z46862 (bglS), Z29076 (eglS) and Z234519 (xynA).
the sacA–purA region of the B. subtilis chromosome and is closely linked to the but locus (O’Kane et al., 1985; Borris et al., 1986). An ORF, designated bglT, precedes the bglS gene. The bglT gene product shares striking similarity with anti-terminator proteins such as those encoded by Escherichia coli bglG, and B. subtilis sacY and sacT genes (Lindner et al., 1993). The position of egl on the B. subtilis chromosome is not known. Strains harbouring mutations within the bgl gene retain about 5–10% of wild-type activity against 1,3-1,4-β-glucan, suggesting that enzymes other than the bgl gene product might be involved in degrading mixed linked β-glucans (Borris et al., 1986).

xynA and xynB mutants (deficient in extracellular xylanase and cell-bound β-xylanidase, respectively) unable to use xylan as sole carbon source were mapped at 48º on the B. subtilis chromosome (Roncero, 1983). The xynB and xyl genes of the xylose regulon are clustered in a 7.5 kb segment of the chromosome of B. subtilis (Hastrup, 1988). Azavedo et al. (1993) mapped xyl at about 173º by probing with a YAC library. Studies by S. A. Zahler, cited by Azavedo et al. (1993), also indicate that the xylose regulon maps near thyA (168º) and citB (173º). Therefore, we must assume that the xynA locus is in fact far away from the clustered xynC–xynB and xylA–xylB operons or the mapping data for xynA need to be corrected.

To analyse degradation of cellulose and hemicelluloses by B. subtilis strain 168, xynA, bglS and eglS were cloned and characterized. The genes encoding extracellular hemicellulases and cellulases are not clustered but rather map at different sites on the chromosome. Egl1·Bgl1 double mutants, constructed in this study, suggest that only the bglS and eglS gene products are involved in β-glucan degradation in B. subtilis. The β-glucanase-negative strains might be used to study expression of genes cloned from other Gram-positives.

**METHODS**

**Bacterial strains and plasmids.** A list of Bacillus subtilis strains and plasmids used in this study is given in Table 1.

**Detection of extracellular endo-β-glucanases and xylanases.** For the detection of enzyme activity on plates, LB agar was supplemented with 0.05% lichenan, 0.2% cellulose, and 0.7% oat xylan. After growth at 37 ºC, plates were stained by flooding with 0.1% Congo red: clear haloes around colonies are indicative of β-glucanase activity. Improved clear zones were obtained by treatment of stained agar plates with 1 M NaCl. Agar plates containing Azurine-Crosslinked (AZCL) polysaccharides (Megazyme) were prepared by adding 0.2% substrate to LB agar. Extracellular enzyme activity was indicated by precipitation of the substrate particles around the colonies.

Cellular fractions of E. coli transformed by recombinant plasmids and supernatants of various B. subtilis strains were subjected to activity staining of endo-β-glucanases after SDS-PAGE. The fractionation of extracellular, periplasmic and cytoplasmic endoglucanases was carried out by the method of Cornelis (Cornelis et al., 1982). SDS-PAGE was done in 15% (w/v) acrylamide gel. Zymograms were prepared using 0.5% CM-cellulose, 0.5% oat spelt xylan, and 0.3% lichenan that had been copolymerized with polyacrylamide. After running, SDS was removed by repeated washing with ethanol/water (1:1, v/v). The gels were equilibrated in 50 mM sodium acetate buffer, pH 6.0, incubated for 1 h at 37 ºC, and stained with 0.5% Congo red. Clear zones were improved with 0.5 M NaCl in 10 mM Tris/HCl, pH 8.

Assays for Vmax were performed with purified protein samples in 50 mM sodium acetate buffer, pH 6.0, supplemented with 10 mM CaCl2 as described by Olsen et al. (1991). Lichenan (29%), oat spelt xylan (0.5%), oat substrates and birch wood xylan (0.5%) were used as substrates. One unit of enzyme activity is defined as the amount of enzyme producing 1 μmol reducing sugar (as glucose equivalents) per minute. Alternatively, enzyme activity was measured using AZCL barley β-glucan, AZCL HE-cellulose, and AZCL xylan from oat spelt as substrate. AZCL polysaccharide (5 mg) in 20 mM sodium acetate buffer, pH 6.0, supplemented with 10 mM CaCl2 and 50 μg bovine serum albumin was shaken with the enzyme for 20 min at 50 ºC. To stop the reaction, 2 ml ethanol was added and the optical density was measured at 595 nm. One unit was defined as the amount of enzyme giving an OD595 of 1.0 min-1.

**Detection of β-galactosidase.** When β-galactosidase β-complementation was assayed, either X-Gal (60 μg ml-1) or the fluorogenic substrate 4-methylumbelliferyl-β-D-galactopyranoside (MUG) was used. MUG (10 mg ml-1 in DMSO) was sprayed onto plates after colonies had formed. Fluorescence produced by hydrolysis of MUG reflects the actual activity of β-galactosidase present in bacteria.

**Cloning and sequencing of the egl and bgl genes.** Clones harbouring the bglS and eglS genes were isolated from a genomic library of B. subtilis 168 cloned in phage λg47.1 (Putzer et al., 1990). DNA from phage clones exhibited β-glucan hydrolysing activity was isolated, digested by HindIII and subcloned into vector pBR322 (Bolivar et al., 1977). Clones hydrolysing CM-cellulose (eglS) and lichenan (bglS) were isolated. Isolated plasmids were shown to direct expression of eglS (pBR36) and bglS (pBR31). Another approach to isolating genes encoding β-glucan hydrolases used vector plasmid pBR322. Chromosomal DNA isolated from B. subtilis 168 was partially digested by SauIII and size-fractionated by agarose gel electrophoresis. DNA fragments of 2–10 kb in size were ligated into pBR322 linearized by BamHI and dephosphorylated by alkaline phosphatase. E. coli DH5α cells transformed with the ligation mixture were screened for CMCase activity as described above. Plasmid pAG1 directs synthesis of B. subtilis cellulase in E. coli.

DNA sequencing was done by dye-deoxy-chain termination reactions (Sanger et al., 1977). Fragments from plasmids pAG1 and pRB36 were created by using convenient restriction sites and subcloned into the appropriately digested and dephosphorylated vectors pTZ19R and pTZ18R (Mead et al., 1986). Double-stranded recombinant plasmid DNAs were used as templates.

**Amplification and sequencing of the xynA gene.** xynA-specific DNA was amplified by PCR from B. subtilis 168 chromosomal DNA. DNA from B. subtilis 168 was prepared by the method of Saito & Miura (1963). Reaction mixtures of 50 μl contained 100 ng PvuII-cleaved DNA, 50 pmol of each oligonucleotide primer, 25 mM Tris/HCl, pH 8.5, 50 mM KCl, 3 mM MgCl2, 0.5 mM of each nucleotide triphosphate (NTP) and 2 μl Taq polymerase. The sequences of the primers were deduced from the published sequence of the B. subtilis PAPI15 xynA gene (Lindner et al., 1994): xynA/o1, 5’-CTGATTCGATTTAGAAGGAAGG-3’; xynA/o2, 5’-CTGATTAAGGAAGATTCGTTACC-3’. The restriction sites for EcoRI (xynA/o1) and BglII (xynA/o2) within the primer sequence are underlined. Template DNA was denatured at 94 ºC for 1 min, annealed at 55 ºC for 1 min and extended for 2 min at 72 ºC for 30 cycles. The 770 bp PCR product was
digested with *Eco*RI and *Bgl*II. Electrophoresis was done in 1.2% agarose gels (Seakem). The amplified DNA product was precipitated and cloned into pTZ18R digested with appropriate restriction enzymes. The resulting recombinant plasmid directed xylanase synthesis in transformed *E. coli* DH5α cells and was used for sequencing by the chain termination method (Sanger et al., 1977). To get sequence information for both strands, subfragments prepared by several restriction enzymes were used as templates for DNA sequencing.

**Gene expression and purification of enzymes.** *E. coli* DH5α cells transformed with recombinant plasmids encoding xylanase (Xyn), lichenase (Bgl) or CMCase (Egl) were grown in standard LB medium containing ampicillin (100 µg ml⁻¹) for 20 h. 1,3,1,4-endo-β-glucanase in the periplasmic space was liberated by osmotic shock as described by Cornelis et al. (1982), resuspended in sodium acetate buffer, pH 4.5, before loading onto a CM-Sepharose CL-6B column. Xylanase was prepared from the supernatant by precipitating the enzyme was equilibrated against acetate buffer, pH 4.5, before loading onto a CM-Sepharose CL-6B column.

**Table 1. Strains and plasmids**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Characteristics</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>trpC2</td>
<td>BGSC, Ohio, USA</td>
</tr>
</tbody>
</table>
| 6GM15 | trpC2 tyr met his ara rib (r_
| | laciZAM15 Km⁸ | Haima et al. (1990) |
| DB104 | his nprR2 nprE18 Δapr-A3 | Kawamura & Doi (1984) |
| MW8 | his nprR2 nprE18 Δapr-A3 ΔeglS102 | This work |
| MW9 | his nprR2 nprE18 Δapr-A3 ΔeglS102 ΔeglS55 | This work |
| MW10 | his nprR2 nprE18 Δapr-A3 ΔeglS102 ΔeglT bglSRV | This work |
| MW11 | his nprR2 nprE18 Δapr-A3 ΔeglS102 ΔeglT bglSRV ara rib (r_
| | laciZAM15 Km⁸ | This work |
| MW12 | his nprR2 nprE18 Δapr-A3 ΔeglS102 ΔeglT bglSRV trpC2 ara rib (r_
| | laciZAM15 Km⁸ | This work |
| MW13 | his nprR2 nprE18 Δapr-A3 ΔeglS102 ΔeglT bglSRV trpC2 tyr ara rib (r_
| | laciZAM15 Km⁸ | This work |
| MW14 | his nprR2 nprE18 Δapr-A3 ΔeglS55 | This work |
| MW15 | his nprR2 nprE18 Δapr-A3 ΔeglS102 ΔeglT bglSRV ΔxynA Cm⁸ | This work |
| **YACs** | | |
| YAC 12-5 | 130 kb YAC insert with thyA xyl citB (map position 165⁰) | P. Serrow, INRA, Jouy-en-Josas, France |
| YAC 15-37 | 130 kb YAC insert with citB (map position 169⁰) | |
| YAC 15-19 | 175 kb YAC insert with gltA terC absA (map position 173⁰) | |
| **Plasmid** | | |
| pHIP9 | Cm⁸ Er³, cat-85::lacZα, pTA1060-pUC9 derivative | Haima et al. (1990) |
| pBR31 | pBR322 containing a 5-6 kb HindIII fragment of *egl* of *B. subtilis* | This work |
| pBR33 | pBR322 containing a 2-8 kb HindIII-SphI fragment of *eglS* | This work |
| pAG1 | pBR322 containing a 3-6 kb fragment of *egl* of *B. subtilis* | This work |
| pAG2 | pBR322 containing a 1-9 kb fragment of *egl* of *B. subtilis* | This work |
| pBR36 | pBR322 containing a 2-8 kb HindIII of *egl* of *B. subtilis* | This work |
| pBR37 | pUC19 containing a 2-8 kb HindIII of *egl* of *B. subtilis* | This work |
| pHV60 | Ap⁰ Tc⁰ Cm⁸, pBR322 derivative, cat gene from pC194 | Michel et al. (1983) |
| pMW1 | pHV60 containing a 2-8 kb HindIII-SphI fragment of pBR33 | This work |
| pMW2 | pHV60 containing a 4-3 kb EcoRV-SalI fragment of pAG1 | This work |
| pMW3 | pMW1 containing a 0-7 kb deletion within *eglS* | This work |
| pMW4 | pMW1 containing a 1-2 kb deletion within the *bgl*-eglS region | This work |
| pMW5 | pMW2 containing a 0-7 kb deletion within *eglS* | This work |
| pMW6 | pBR322 containing 770 bp of xynA | This work |
| pMW7 | pTZ18R containing 770 bp of xynA | This work |
| pMW8 | pHV60 containing an internal *PreI*-SalI fragment of xynA | This work |

**Transformation and mapping procedures.** *E. coli* cells were grown and prepared for transformation as described by Ledeborg & Cohen (1974) and competent *B. subtilis* cells were transformed as described previously (Borriss et al., 1986). PBS1 transduction was performed with lysates from a set of *B. subtilis* strains containing transposon *Tn917* insertions (Vandeyar & Zahler, 1986). The recipient was the double mutant strain MW10 devoid of lichenase and cellulase activity. Em⁸ transductants were scored for their ability to hydrolyse lichenase and CM-cellulose. To map the *eglS* gene more precisely, a lysate

**graphy using Sephacryl S-200 (Pharmacia) before analysis by SDS-PAGE. CMCase was purified in a similar way except that the enzyme was equilibrated against acetate buffer, pH 4.5, before loading onto a CM-Sepharose CL-6B column.**
of PBS1 grown on MW10 was used to infect the reference strain 1A6 (trpD1 pyrD1 thyA1 thyB1 trpC2) and 1A7 (gltA292 trpC2; Dedonder et al., 1977). Transductants were scored for their ability to hydrolyse CM-cellulose. *synA* was mapped essentially in the same way except that strain MW15, devoid of xylanase, lichenase and cellulase activity, was the recipient. A PBS1 lysate made on MW15 was used to infect strains 1A6 and 1A7. The ability of the transductants to hydrolyse AZCL xylan was scored.

Digoxigenin-labelled *synA*, *bglS* and *eglS* gene probes were used to hybridize an ordered collection of YAC clones containing more than 98% of the whole *B. subtilis* genome (Azevedo et al., 1993). A nylon membrane containing the whole set of YAC clones was prehybridized for 2 h at 42 °C in hybridization solution, followed by hybridization with the probes overnight at 42 °C in the same solution. YAC clones hybridizing with *egl* and *bgl* were detected after washing twice in 50% (v/v) formamide, 0·1% SDS, 0·36 M NaCl, 20 mM sodium phosphate, 2 mM EDTA for 20 min at 42 °C and once in 18 mM NaCl, 0·1% SDS for 20 min at 42 °C. The isolation of YAC DNA used for Southern hybridization is described in the accompanying paper (Tam & Borris, 1995).

**Strain deposition.** *Bacillus* strains MW10 (1A751) and MW12 (1A752) have been deposited in the BGSC, Ohio, USA.

**RESULTS**

**Deletion of the *bgl* gene**

To facilitate construction of strains with a deleted *bglS* gene, recombinant phages hydrolysing lichenan were isolated from a 147·1 library. A 5·6 kb *HindIII* DNA fragment isolated from 147·1-Lic1 directs lichenan-degrading activity. The fragment was cloned into pBR322. *E. coli* DH5α cells transformed with the resulting plasmid pRB31 hydrolysed lichenan but were not active against CM-cellulose. Restriction enzyme analysis and hybridization with the *bglS* gene previously cloned from *B. subtilis* 168 (Borris et al., 1986) revealed that the 5·6 kb fragment contains the *bglS* gene (Fig. 1a). The nucleotide sequence of the *bglS* gene was determined. The deduced amino acid sequence was found to be identical in 234 out of 242 residues with that reported for *B. subtilis* 168 (Borriss et al., 1995).

To construct an integration plasmid carrying a defective *bglS* gene, a 1·86 kb *EcoRI–3′pbl* fragment carrying the entire sequence of *bglS* was blunt-ended and cloned into the unique *EcoRV* site of vector pHV60. The resultant plasmid, pMW1, was linearized with *EcoRV* and treated with *Bal31* exonuclease. Deletions of 400–900 bp were obtained. After religation, plasmid pMW3, carrying a deletion within the *bglS* gene of about 700 bp (Δ*glS*55), was selected for further studies. Another deletion (Δ*bgl∗T/*bglS∗V*) within the 2·8 kb *HindIII–Spbl* fragment was obtained by removing the 1·2 kb *EcoRV* fragment containing the 3′ part of *bglT* and the 5′ half of *bglS* (Fig. 1a).

**Structure of the *eglS* gene and biochemical properties of its product**

The *eglS* gene was cloned by screening the phage 147·1 library on agar plates containing 0·2% CM-cellulose (see Methods). Different DNA fragments expressing the *eglS* gene were subcloned into pBR322. Plasmids pAG1 and pBR36 contain overlapping DNA fragments (Fig. 1b). Restriction analysis indicated a high degree of similarity to a DNA fragment containing the endo-1,4-β-glucanase gene from *B. subtilis* PAP115. The nucleotide sequences of the *eglS* gene and its flanking regions were determined. Two ORFs were found within a stretch of 3500 bp. One of them, extending from 1380 to 2876 encodes a protein of 499 amino acid residues. The predicted protein with a molecular mass of 55287 Da was very similar to those of other extracellular cellulases already cloned from other *B. subtilis* strains. In particular, the amino acid sequence of EglS was found to be identical to that of the *eglS* gene product of *B. subtilis* PAP115 but does not share any similarity with the *bglS* gene product.

The mature protein, lacking its signal peptide, consists of 470 amino acid residues with a molecular mass of 52264 Da. Several cellulase isoenzymes with apparent molecular masses ranging from 49 kDa to 31·5 kDa were detected in zymograms of *E. coli* strains transformed by *eglS*-bearing plasmids pRB37 and pAG2 (results not shown). The main activities detected on active gels containing lichenan and CM-cellulose were 35·5 kDa and 33·5 kDa in size (Fig. 4, lane 7). Processing or degradation of the primary translation product, in addition to signal peptide cleavage, has been reported for other endo-1,4-β-glucanases (Lo et al., 1988; Shima et al., 1993).

Biochemical studies performed with the purified product of the *eglS* gene revealed important differences in substrate specificity and *V* max compared with the 1,3-1,4-endo-β-glucanase. The enzyme was able to degrade lichenan, CM-cellulose and AZCL oat xylan. In contrast, the *bglS* gene product only hydrolysed lichenan but not CM-cellulose and xylan. However, specific activity towards the mixed linked 1,3-1,4-β-glucan was much higher in the 1,3-1,4-endo-β-glucanase than in 1,4-endo-β-glucanase (Table 2).

A second but incomplete ORF, *orfX*, encoding more than 358 amino acid residues, was identified 5′ of *eglS*. The N-terminal part of *orfX* is interrupted by the left-hand *HindIII* site of the fragment. The two ORFs are separated by a putative termination signal formed by an inverted repeat of 14 nt at position 1095 (ΔG = $-18·2 \text{kcal}$ ($-76·1$ kJ)]).

To construct an integration plasmid carrying a defective *eglS* gene, we followed the strategy described for *bgl*. A DNA fragment carrying the *eglS* gene was recloned into pHV60, linearized by *MluI*, treated with *Bal31* and religated. A clone with a 643 bp deletion within *eglS* (ΔeglS102) was chosen for integration into the chromosome of *B. subtilis* (Fig. 1b).

**Cloning and properties of the *synA* gene**

A 770 bp DNA fragment harbouring the entire *synA* gene sequence was amplified from chromosomal DNA from *B. subtilis* 168 and recloned into vector plasmids pBR322(pMW6) and pTZ18R(pMW7). *E. coli* cells harbouring recombinant plasmids pMW6 or pMW7 conferred xylanase activity. The nucleotide sequence was found to be identical to that from *B. subtilis* PAP115 (Paice
Enzyme activities were calculated from the results of three independent measurements.

### Table 2. Substrate specificities of endo-1,4-β-glucanase (Egl) and endo-1,4-β-xylanase (XynA)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Bgl (Units)</th>
<th>Egl (Units)</th>
<th>XynA (Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lichenan</td>
<td>2600*</td>
<td>368*</td>
<td>NM</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
</tr>
<tr>
<td>Oat spelt xylan</td>
<td>NM</td>
<td>45*</td>
<td>316*</td>
</tr>
<tr>
<td>Birch wood xylan</td>
<td>NM</td>
<td>NM</td>
<td>228*</td>
</tr>
<tr>
<td>AZCL β-glucan</td>
<td>100†</td>
<td>5†</td>
<td>NM</td>
</tr>
<tr>
<td>AZCL HE-cellulose</td>
<td>NM</td>
<td>20†</td>
<td>NM</td>
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<tr>
<td>AZCL xylan</td>
<td>NM</td>
<td>0.05†</td>
<td>234†</td>
</tr>
</tbody>
</table>

NM, not measurable.
*Unit defined as 1 pmol reducing sugar min⁻¹ (mg purified enzyme)⁻¹.
†Unit defined as 1 OD₉₅₀ unit min⁻¹ (mg purified enzyme)⁻¹.

Transformation of DB104 with integrating plasmid pMW5 carrying the deleted egls gene with the flanking sequences (Δegls102) yielded transformants with two different phenotypes: most of the transformants were CmR EglS⁺ but about 10% were found to be CmR, EglS⁻. The EglS⁻ phenotype could not result from a single crossover event between homologous regions because the egls gene would not be interrupted. Most likely the EglS⁻ phenotype derives from a double crossover replacement between the homologous chromosomal region and concatemer of pMW5 (Stahl & Ferrari, 1984). The plasmid phenotype is unstable because of flanking directly repeated DNA. To excise plasmid DNA, CmR EglS⁺ transformants were cultivated for about 10 generations in antibiotic-free medium. One cured CmS clone, designated MW8, with a stable EglS⁻ phenotype was selected.

Transformation of MW8 with the integrative plasmid pMW4 carrying the deleted egls gene yielded only CmR BglS⁺ clones. The BglS⁺ phenotype was not detected. This result might be due to the size of the 1-2 kb deletion introduced in the bglt/bgls region. Clones with the CmR BglS⁻ phenotype were obtained after cultivation in the absence of antibiotic selection and one of them, named
MW10, was chosen for further studies. Another deletion within the bglS gene was introduced using integrative plasmid pMW3. The CmR Bgl- phenotype was not observed after transformation into MW8. However, after cultivation without selection pressure, stable CmR Bgl- clones could be isolated. One of these was designated MW9. Southern hybridization verified that the transformed strains harbour the deleted bglS and eglS genes (Fig. 2).

Another strategy was used to construct strains deficient in xylanase activity. The central 382 bp PstI-Sal fragment isolated from the xynA gene was cloned into integrative plasmid pHV60, digested with appropriate restriction enzymes and used to transform strain MW10 (lacking cellulase and lichenase activity). CmR clones were checked for deficiency in xylanase activity on AZCL-xylan-containing plates. Southern hybridization revealed that the xylanase-deficient strain MW15 contains two deleted copies of xynA on the chromosome (results not shown).

**Mutant strains with the β-galactosidase α-complementation systems**

To use the versatile β-galactosidase α-complementation system developed for *B. subtilis* (Haima et al., 1990) the lacZAM15 gene from 6GM15 was introduced by conjugation into strain MW10 (Egl- Bgl-). The resulting strains MW11–MW13 would exhibit a blue phenotype on X-Gal agar plates if transformed by plasmid pHPS9 (lacZα), but only light blue colonies were detected after 2 d at 37 °C. Using MUG as substrate, β-galactosidase-producing clones could be detected after 1 d at 37 °C.

To test the usefulness of the α-complementation system for direct cloning of recombinants in *B. subtilis*, a cloning experiment with the 1,3-1,4-β-glucanase gene from *B. amyloliquefaciens* (bglA) was performed. A 3.6 kb EcoRI DNA fragment harbouring the bglA gene (Borriss et al., 1985) was ligated to EcoRI-cleaved pHPS9 DNA. The ligation mixture was used to transform competent MW12 cells, and transformants were selected on plates containing Cm, X-Gal and lichenan. After 2 d, light blue and white CmR colonies could be distinguished. Staining with Congo red revealed that only white colonies expressed β-glucanase activity on lichenan agar. As expected, the white clones contained recombinant plasmids with the 3.6 kb DNA fragment, whereas the blue clones contained only the religated vector pHPS9.

**Characterization of mutant strains**

Expression of β-glucan hydrolases was examined on agar plates supplemented with lichenan and CM-cellulose. Strain MW8, deficient in extracellular 1,4-endo-β-glucanase (EglS), did not hydrolyse CM-cellulose agar but was able to degrade lichenan. Double mutants harbouring EglS and AbglS lacked 1,3-1,4-β-glucan hydrolysing activity, whereas strain MW14 with AbglS55 hydrolysed CM-cellulose just like the parental strain DB104. In addition, MW14 was slightly active towards lichenan, suggesting that the eglS gene product is able to hydrolyse mixed linked β-glucans (Fig. 3). MW15 harbouring ΔeglS, ΔbglS and ΔxynA was not able to hydrolyse lichenan, CM-cellulose or AZCL xylan. All strains were capable of
Fig. 3. Production of endoglucanases from mutant and wild type B. subtilis 168. Relevant genotypes of mutant strains: AeglSI02 (MW8), AeglSI02 AbglS55 (MW9), AeglSI02 Abgl bglSRV (MW10) and AbglS55 (MW14). Strains were spotted on a LB agar plate containing either CM-cellulose or lichenan. The plates were incubated at 37°C for 24 h and stained with Congo red. Note: this picture has been electronically imaged.

hydrolysing AZCL galactomannan but unable to degrade AZCL pachyman and AZCL xyloglucan (Table 3). The presence of β-glucan hydrolysing enzymes was also checked by the zymogram technique (Fig. 4). Lichenase with an apparent molecular mass of 24 kDa was detected in DB104 (wild type) and MW8 (AeglS); cellulase isoenzymes with an apparent molecular mass of 34–36 kDa were detected in DB104 and MW14 (AbglS). The apparent molecular mass of cellulase isoenzymes is much less than that deduced from the nucleotide sequence of the B. subtilis eglS gene. As in the case of the cloned eglS gene in E. coli, this might be due to processing and degradation of primary translation products. Lo et al. (1988) reported that the 52.2 kDa cellulase of B. subtilis PAP115 is cleaved progressively to a product of about 32 kDa in its native host.

bglS, eglS and xynA map in different regions of the B. subtilis chromosome

With lysates prepared from 1A645 (zei82::Tn917) containing a silent Tn917 insertion at 342°, 87.3% cotransduction of the bglS marker was observed. The bglS locus has previously been mapped to around 340° on the B. subtilis chromosome (O’Kane et al., 1985; Borriss et al., 1986).

A high frequency of cotransduction of eglS was found with lysates containing a Tn917 insertion close to gltA (map position 177°, Anagnostopoulos et al., 1993) prepared from 1A634 (zei82::Tn917). To map the eglS gene more precisely, reference recipients of the Dedonder kit were transduced by PBS1 grown in MW10 (AeglS, AbglS), and the resulting recombinants were checked for linkage to the eglS locus. The results shown in Fig. 5 demonstrate linkage of eglS to thyA (168°, Tam & Borriss, 1995) and gltA (177°), indicating a position of around 170° on the circular chromosomal map of B. subtilis.

Cotransfer of xynA was also observed with lysates prepared from 1A634 (zei82::Tn917) using strain MW15 (Aegl Abgl AxynA) as recipient. Of the primary EmR transductants, 27% exhibited the XynA+ phenotype as checked by plating on AZCL xylan agar. Dedonder kit strains 1A6 (thyA) and IA7 (gltA) were used as recipients for donor lysates prepared from MW15. Six percent of ThyA+ and 63% of GltA+ transductants were XynA+, indicating cotransfer of the xynA marker. The genetic map compiled from data obtained by PBS1 transduction demonstrates that eglS and xynA are located within the thyA–gltA region at about 170° and 175° on the circular B. subtilis chromosome, respectively (Fig. 5).

In addition, a YAC library (Azevedo et al., 1993) was used to probe DNA fragments containing the eglS and bglS genes with an ordered collection of contiguous segments.
Apparent molecular masses are indicated. (a) SDS-PAGE gel with CM-cellulose. Lanes: 1, 0.5 µg purified 1,3-1,4-endo-p-glucanase; 2-6, supernatants from DB104 (2), MW8 (3), MW9 (4), MW10 (5) and MW14 (6); 7, 0.6 µg purified protein from E. coli(pAG2) cells expressing B. subtilis 1,4-endo-p-glucanase (lane 1) and 1,4-endo-p-xylanase (lane 2) contains two lichenan hydrolysing activities with apparent molecular masses of 24 kDa (lichenase) and 33 kDa (cellulase). Lanes were loaded as indicated in (a). Note that the supernatant of DB104 has been electronically imaged. (b) SDS-PAGE gel with lichenan. Lanes were loaded as indicated in (a). Note: this picture is a picture of the gel, not a description of the gel.

**DISCUSSION**

Numerous enzymes hydrolysing cellulose and different hemicelluloses as mixed linked β-glucan, xylan and galactomannan are secreted by B. subtilis (Table 3). Genes encoding 1,3-1,4-β-glucanase (bg1S), 1,4-β-glucanase (eglS) and 1,4-β-xylanase (xylanA) have been cloned previously from different B. subtilis strains, especially from industrial strain PAP115, and structurally characterized. We cloned the bg1S, eglS and xylanA genes from B. subtilis 168. The bg1S gene hybridized with a 190 kb fragment carrying the but locus (designated clone 13-2), confirming previous results obtained by PBS1-mediated transduction. Interestingly, a gene designated bg1A which encodes phospho-β-glucosidase hybridized in the region of overlap of YAC clones 13-2 and 11-237 between but (335°) and gnt (344°) (Zhang & Aronson, 1993).

Hybridizing signals from the eglS gene fragment were obtained with YAC clones 12-5 and 15-37 which form overlapping contigs of the B. subtilis chromosome from 165° to 173° and cover the thyA, xyl and citB loci as well (Azevedo et al., 1993). Southern hybridization of eglS probed with EcoRI-digested DNA prepared from different YACs verified the presence of eglS on overlapping contigs YAC 12-5 and 15-37 (Fig. 5).

Different results were obtained when the YAC library was used to probe for xynA. EcoRI-digested DNA prepared from YAC 15-19 carrying gntA but not YAC 12-5 and 15-37, which contain the eglS gene, hybridized with xynA. Thus, the results obtained by genetic and physical mapping of eglS and xynA indicate that both genes are located within the region between thyA (168°) and gntA (177°) which is covered by YAC contigs 12-5, 15-37 and 15-19. The map shows the gene order thyA–eglS–xynA–gntA (Fig. 5). xynA, which is located at about 175°, is clearly separated from eglS (170°) and the xyl–xynA cluster, which has been mapped at 168° on the B. subtilis chromosome (Azevedo et al., 1993).

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**Fig. 4.** Production of endoglucanases by mutant and wild-type B. subtilis 168 strains. Cells were cultured in LB medium at 37 °C for 20 h. Supernatants were concentrated by ultrafiltration and applied to SDS-polyacrylamide gels containing either lichenan or CM-cellulose. The gels were stained with Congo red.

**Fig. 5.** Linkage relationships of thyA, eglS, zei82::Tn917, xynA and gntA markers. Numerical values represent recombination indices calculated from at least two separate transduction experiments. Arrows point from the selected to the unselected marker. YAC clones 12-5, 15-37 and 15-19 correspond to the thyA–gntA region of the B. subtilis chromosome. Hybridization signals obtained for thyA, eglS and xynA with the YAC clones are indicated by bold arrows in the bottom part of the figure. Probing of the thyA gene fragments with DNA isolated from YAC 12-5 is described in the accompanying paper (Tam & Borriss, 1995).
As reported by Murphy et al. (1984), bglS encodes a protein with a molecular mass of 27 338 Da. Processing of the 28 amino acid residue signal peptide results in a protein of about 24 kDa (Yuuki et al., 1989). According to its primary sequence, the gene belongs to family 16 of glycosyl hydrolases formed by bacterial endo-1,3-1,4-β-glucanases (Henrissat & Bairoch, 1993). The substrate specificity of endo-1,3-1,4-β-glucanase is restricted to mixed linked β-glucans such as lichenan or barley β-glucan. No other hemicellulosic or cellullosic substrates are hydrolysed (Table 2).

The B. subtilis eglS gene product (endo-1,4-β-glucanase) has been classified into family 9 of the glycosyl hydrolases (Henrissat, 1991). The enzyme is highly similar to other bacterial endo-1,4-β-glucanases and is able to hydrolyse CM-cellulose and mixed linked β-glucans as well. The specific lichenan hydrolysing activity of 368 U (mg protein)⁻¹ was much less than the specific activity of 2600 U mg⁻¹ determined for endo-1,3-1,4-β-glucanase, however. In addition, EglS has a very low but detectable activity on AZCL xylan (Table 2).

Despite the weak xylan hydrolysing activity detected in EglS, the xynA gene product makes a major contribution to xylan-degrading activity in B. subtilis 168. The enzyme shares more than 99% sequence identity with xylanases from B. subtilis PAP115 and B. circulans and has been grouped into family 11 comprising bacterial and fungal endo-1,4-β-xylanases (EC 3.2.1.8; Henrissat, 1991). The enzyme is active against oat spelt xylan and birchwood xylan virtually in the same range, but does not show any activity against the other substrates tested in this study (Table 2).

The gene replacement technique (Stahl & Ferrari, 1984) was used to introduce permanent chromosomal deletions into the eglS and bglS structural genes in B. subtilis DB104. Strains MW10-12 carrying deletions in both genes (ΔeglS ΔbglS) were without detectable activity towards lichenan and CM-cellulose, suggesting that the minor β-glucanase responsible for about 5-10% of total 1,3,1,4-β-glucan hydrolysing activity found in bglS mutant strains of B. subtilis 168 (Borriss et al., 1986) represent processed products of the eglS gene. Biochemical analysis performed with the purified products of bglS and eglS suggests that the 1,3,1,4-β-glucanase degrades mixed linked β-glucans much faster than the 1,4-β-glucanase (see above). The activity of lichenanase, BglS, was found to be seven times higher on lichenan and about 20 times higher on AZCL β-glucan than that of CMCase (EglS; Table 2). Hence, both genes seem to be expressed to a similar level in B. subtilis, but differ in their capacity to hydrolyse mixed linked β-glucans.

Strain MW15 (ΔbglS ΔeglS ΔxynA), constructed by disruption of the xynA gene in strain MW10, was devoid of activity towards lichenan, CM-cellulose and xylan (Table 3). Thus, our results obtained with the deletion mutants strongly suggest that EglS, BglS and XynA are the only enzymes involved in CM-cellulose, 1,3-1,4-β-glucan and xylan hydrolysis in B. subtilis.

The bglS genes encoding cellulases and hemicellulases are possibly part of a global carbon catabolite repression regulon governed by an unidentified trans-acting factor (Krüger et al., 1993). Results obtained by PBS1 transduction and hybridization with contiguous DNA fragments cloned in a YAC library showed that the two genes encoding endo-β-glucan hydrolyses are very distant from each other on the B. subtilis chromosome. The eglS gene was genetically mapped close to the bglS locus at 170°. Southern hybridization performed with DNA from selected YAC clones confirmed the results of transduction analysis. The eglS gene was detected within the overlapping region of two contigs sharing the citB gene region. Similarly, the genetic position of bglS determined by PBS1-mediated transduction at about 340°, adjacent to but loci, was verified by hybridization of bglS DNA with YAC 13-2 containing the but operon.

The same combined genetic and physical approach has proven useful for mapping of the xynA gene. Phage PBS1-mediated transduction with strains harbouring deletions within xynA and the β-glucanase-encoding eglS and bglS genes places xynA at about 175° close to the gltA locus. This contradicts the findings of Roncero (1983) showing that xynA and xynB are clustered at the very distant map position of 48° close to gtaB.

A gene cluster with xylA and xynC-XynB genes was located near bglS at 168° (Hastrup, 1988). The xylA gene was also physically mapped on YAC 12-5 harbouring the bglS gene (Azevedo et al., 1993). To clarify this situation we probed the cloned xynA fragment with the YAC library and with DNA from different contigs covering the region between bglS and gltA. Only YAC 15-19 containing the gltA locus hybridized with xynA, confirming the results of our transduction mapping.

In summary, the three genes described in this study are distinct in structure, chromosomal location and substrate specificity of their respective products. We have been able to show that no other genes direct β-glucan and xylan degradation in B. subtilis. The mutants deficient in endo-β-glucanases and xylanase described here may well prove useful for secretion of heterologous β-glucan hydrolases and xylanases. In addition, direct cloning of genes encoding β-glucan hydrolases might be facilitated by using the α-complementation system introduced into strain MW12.

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