Genes encoding xylan and $\beta$-glucan hydrolysing enzymes in \textit{Bacillus subtilis}: characterization, mapping and construction of strains deficient in lichenase, cellulase and xylanase

Monika Wolf, Attila Geczi, Ortwin Simon and Rainer Borriss

INTRODUCTION

Cellulose (insoluble fibres of $\beta$-1,4-glucan) and hemicellulose (non-cellulosic polysaccharides including glucans, mannans and xylans) are the major constituents of plant cell walls. $\beta$-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-$\beta$-glucans form the major part of cell walls of cereals like oat and barley. The aerobic soil bacterium \textit{Bacillus subtilis} secretes different hydrolases enabling this organism to use external cellulolytic and hemicellulosic substrates. Three types of $\beta$-glucan endohydrolase able to degrade 1,3,1,4-$\beta$-glucans but distinguished by fine differences in substrate specificity are known. Two of them, endo-$\beta$-1,4-glucanase (eglS) and endo-$\beta$-1,3,1,4-glucanase (bglS) were isolated from a genomic library of \textit{B. subtilis} 168. The sequences of \textit{xynA} and \textit{eglS} were identical to those of the xylanase and cellulase genes from \textit{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 \textit{eglS}, \textit{bglS} and \textit{xynA} genes of \textit{B. subtilis} 168. Strains without any detectable activity against xylan (Xyn), carboxymethylcellulose (Egl) or mixed linked $\beta$-1,3,1,4-glucan (Egl$^{-}$Bgl$^{-}$) were obtained. The genes were mapped at 170$^\circ$ (eglS), 175$^\circ$ (xynA) and 340$^\circ$ (bglS) on the \textit{B. subtilis} chromosome.

Keywords: \textit{Bacillus subtilis}, genome sequencing, gene mapping, xylanase, cellulase, lichenase

The gene encoding extracellular xylanase \textit{(xynA)} was amplified as a 770 bp DNA fragment from \textit{Bacillus subtilis} 168 chromosomal DNA by PCR. The genes encoding endo-$\beta$-1,4-glucanase (eglS) and endo-$\beta$-1,3,1,4-glucanase (bglS) were isolated from a genomic library of \textit{B. subtilis} 168. The sequences of \textit{xynA} and \textit{eglS} were identical to those of the xylanase and cellulase genes from \textit{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 \textit{eglS}, \textit{bglS} and \textit{xynA} genes of \textit{B. subtilis} 168. Strains without any detectable activity against xylan (Xyn), carboxymethylcellulose (Egl$^{-}$) or mixed linked $\beta$-1,3,1,4-glucan (Egl$^{-}$Bgl$^{-}$) were obtained. The genes were mapped at 170$^\circ$ (eglS), 175$^\circ$ (xynA) and 340$^\circ$ (bglS) on the \textit{B. subtilis} chromosome.

Abbreviations: AZCL, Azurine-Crosslinked; CMCase, carboxymethylcellulase; MUG, 4-methylumbelliferyl-$\beta$-D-galactopyranoside.

The EMBL accession numbers for the nucleotide sequences reported in this paper are Z46862 (bgl), Z29076 (egl) and Z34519 (xynA).
the $\text{sacA} - \text{purA}$ region of the $\text{B. subtilis}$ chromosome and is closely linked to the $\text{bgl}$ locus (O’Kane et al., 1985; Borris et al., 1986). An ORF, designated $\text{bglT}$, precedes the $\text{bglS}$ gene. The $\text{bglT}$ gene product shares striking similarity with anti-terminator proteins such as those encoded by $\text{Escherichia coli}$ $\text{bglG}$, and $\text{B. subtilis}$ $\text{sacY}$ and $\text{sacT}$ genes (Lindner et al., 1993). The position of $\text{egf}$ on the $\text{B. subtilis}$ chromosome is not known. Strains harbouring mutations within the $\text{bgl}$ gene retain about 5-10% of wild-type activity against 1,3-1,4-$\beta$-glucan, suggesting that enzymes other than the $\text{bgl}$ gene product might be involved in degrading mixed linked $\beta$-glucans (Boriss et al., 1986).

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

To analyse degradation of cellulose and hemicelluloses by $\text{B. subtilis}$ strain 168, $\text{xynA}$, $\text{bglS}$ and $\text{egfS}$ were cloned and characterized. The genes encoding extracellular hemicellulases and cellulase are not clustered but rather map at different sites on the chromosome. $\text{Egl}^+$ $\text{Bgl}^+$ double mutants, constructed in this study, suggest that only the $\text{bglS}$ and $\text{egfS}$ gene products are involved in $\beta$-glucan degradation in $\text{B. subtilis}$. The $\beta$-glucanase-negative strains might be used to study expression of genes cloned from other Gram-positives.

**METHODS**

**Bacterial strains and plasmids.** A list of $\text{Bacillus subtilis}$ strains and plasmids used in this study is given in Table 1.

**Detection of extracellular endo-$\beta$-glucanas and xylanases.** For the detection of enzyme activity on plates, $\text{LB}$ agar was supplemented with 0.05% lichenan, 0.2% cellulose, and 0.7% oat xylan. After growth at 37°C, plates were stained by flooding with 0.1% Congo red: clear haloes around colonies are indicative of $\beta$-glucanase activity. Improved clear zones were obtained by treatment of stained agar plates with 1 M NaCl.

**Assays for $V_{\text{max}}$.** Assays for $V_{\text{max}}$ were performed with purified protein samples in 50 mM sodium acetate buffer, pH 6.0, supplemented with 10 mM CaCl$_2$ as described by Olsen et al. (1991). Lichenan (20%, w/v), CM-cellulose (0.5%), oat spelts and birch wood xylan (0.5%) were used as substrates. One unit of enzyme activity is defined as the amount of enzyme producing 1 pmol reducing sugar (as glucose equivalents) per minute. Alternatively, enzyme activity was measured using AZCL barley $\beta$-glucan, AZCL HE-cellulose, and AZCL xylan from oat spelts as substrate. AZCL polysaccharide (5 mg) in 20 mM sodium acetate buffer, pH 6.0, supplemented with 10 mM CaCl$_2$ and 50 µg bovine serum albumin was shaken with the enzyme for 20 min at 50°C. 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 OD$_{595}$ of 1.0 min$^{-1}$.

**Detection of $\beta$-galactosidase.** When $\beta$-galactosidase activity was assayed, either X-Gal (60 µg ml$^{-1}$) or the fluorogenic substrate 4-methylumbelliferyl-$\beta$-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 $\beta$-galactosidase present in bacteria.

**Cloning and sequencing of the $\text{egf}$ and $\text{bgl}$ genes.** Clones harbouring the $\text{bglS}$ and $\text{egfS}$ genes were isolated from a genomic library of $\text{B. subtilis}$ 168 cloned in phage $\lambda$L47.1 (Putzer et al., 1990). DNA from phage clones exhibited $\beta$-glucan hydrolysing activity was isolated, digested by HindIII and subcloned into vector pBR322 (Bolivar et al., 1977). Clones hydrolysing CM-cellulose (egfS) and lichenan (bglS) were isolated. Selected plasmids were shown to direct expression of $\text{bglS}$ (pRB36) and $\text{egfS}$ (pRB31). Another approach to isolating genes encoding $\beta$-glucan hydrolases used vector plasmid pBR322. Chromosomal DNA isolated from $\text{B. subtilis}$ 168 was partially digested by SauIII A and size-fractionated by agarose gel electrophoresis. DNA fragments of 2–10 kb in size were ligated into pBR322 linearized by BamHII and dephosphorylated by alkaline phosphatase. $\text{E. coli}$ DH5α cells transformed with the ligation mixture were screened for CMCase activity as described above. Plasmid pAG1 directs synthesis of $\text{B. subtilis}$ cellulase in $\text{E. coli}$. 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 $\text{xynA}$ gene.** $\text{xynA}$-specific DNA was amplified by PCR from $\text{B. subtilis}$ 168 chromosomal DNA. DNA from $\text{B. subtilis}$ 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 MgCl$_2$, 0.5 mM of each nucleoside triphosphate (NTP) and 2 U Taq polymerase. The sequences of the primers were deduced from the published sequence of the $\text{B. subtilis}$ PAPIA $\text{xynA}$ gene (Lindner et al., 1994): $\text{xynA/oli1}$, 5’-GGAGATTCGTTGTTATTAATTGTCGAGG-3’; $\text{xynA/oli2}$, 5’-GGCTGAGATAGAAGATGTCGAGG-3’.

The restriction sites for EcoRI ($\text{xynA/oli1}$) and $\text{BglI}$ ($\text{xynA/oli2}$) within the primer sequence are underlined. Template DNA was denatured at 94°C for 1 min, annealed at 55°C for 1 min and extended for 2 min at 72°C for 30 cycles. The 770 bp PCR product was
were used as templates for DNA sequencing. Cells transformed with recombinant plasmids encoding xylanase was used for sequencing by the chain termination method.

Gene expression and purification of enzymes. E. coli DH5α cells were grown and prepared for transformation as described by Lederberg & Cohen (1974) and competent B. subtilis cells were transformed as described previously (Borriss et al., 1986).

Transformation and mapping procedures. E. coli cells were grown and prepared for transformation as described by Lederberg & 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. EmR transductants were scored for their ability to hydrolyze lichenan and cellulase activity. EmR transductants were scored for their ability to hydrolyze lichenan and cellulase activity.

Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Characteristics</th>
<th>Source/reference</th>
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</thead>
<tbody>
<tr>
<td>B. subtilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>trpC2</td>
<td></td>
</tr>
<tr>
<td>6GM15</td>
<td>trpC2 tyr met his ara rib (r^m^m) lacZAM15 KmR</td>
<td>Haima et al. (1990)</td>
</tr>
<tr>
<td>DB104</td>
<td>his nprR2 nprE18 ΔaprA3</td>
<td>Kawamura &amp; Doi (1984)</td>
</tr>
<tr>
<td>MW8</td>
<td>his nprR2 nprE18 ΔaprA3 ΔeglS102</td>
<td>This work</td>
</tr>
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<td>This work</td>
</tr>
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<td>his nprR2 nprE18 ΔaprA3 ΔeglS102 ΔbglT bglSRV</td>
<td>This work</td>
</tr>
<tr>
<td>MW11</td>
<td>his nprR2 nprE18 ΔaprA3 ΔeglS102 ΔbglT bglSRV ara rib (r^m^m) lacZAM15 KmR</td>
<td>This work</td>
</tr>
<tr>
<td>MW12</td>
<td>his nprR2 nprE18 ΔaprA3 ΔeglS102 ΔbglT bglSRV trpC2 ara rib (r^m^m) lacZAM15 KmR</td>
<td>This work</td>
</tr>
<tr>
<td>YAC 12-5</td>
<td>130 kb YAC insert with txyB xyl citB (map position 165°)</td>
<td>P. Serrut, INRA, Jouy-en-Josas, France</td>
</tr>
<tr>
<td>YAC 15-37</td>
<td>130 kb YAC insert with citB (map position 169°)</td>
<td></td>
</tr>
<tr>
<td>YAC 15-19</td>
<td>175 kb YAC insert with gltA terC adhA (map position 173°)</td>
<td></td>
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<tr>
<td>Plasmid</td>
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<td></td>
</tr>
<tr>
<td>pHP50</td>
<td>CmR ErR, cat-86 : lacZa, pTA1050-pUC9 derivative</td>
<td>Haima et al. (1990)</td>
</tr>
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<td>pBR332</td>
<td>pBR322 containing a 56 kb HindIII fragment of bgl of B. subtilis</td>
<td>This work</td>
</tr>
<tr>
<td>pBR335</td>
<td>pBR322 containing a 28 kb HindIII–SpeI fragment of bglS</td>
<td>This work</td>
</tr>
<tr>
<td>pAG1</td>
<td>pBR322 containing a 36 kb fragment of egl of B. subtilis</td>
<td>This work</td>
</tr>
<tr>
<td>pAG2</td>
<td>pBR322 containing a 1-9 kb fragment of egl of B. subtilis</td>
<td>This work</td>
</tr>
<tr>
<td>pBR36</td>
<td>pBR322 containing a 28 kb HindIII of egl of B. subtilis</td>
<td>This work</td>
</tr>
<tr>
<td>pBR37</td>
<td>pUC19 containing a 28 kb HindIII of egl of B. subtilis</td>
<td>This work</td>
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<tr>
<td>pHV60</td>
<td>ApR TrR CmR, pBR322 derivative, cat gene from pC194</td>
<td>Michel et al. (1983)</td>
</tr>
<tr>
<td>pMW1</td>
<td>pHV60 containing a 28 kb HindIII–SpeI fragment of pBR33</td>
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</tr>
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<td>pMW2</td>
<td>pHV60 containing a 43 kb EcoRV–SalI fragment of pAG1</td>
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<td>pMW3</td>
<td>pMW1 containing a 6 kb deletion within bglS</td>
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</tr>
<tr>
<td>pMW4</td>
<td>pMW1 containing a 4 kb deletion within the bgl–bglS region</td>
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<td>pMW5</td>
<td>pMW2 containing a 7 kb deletion within eglS</td>
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<td>pMW6</td>
<td>pBR322 containing 700 bp of xynA</td>
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</tr>
<tr>
<td>pMW7</td>
<td>pTZ218R containing 700 bp of xynA</td>
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</tr>
<tr>
<td>pMW8</td>
<td>pHV60 containing an internal Pfl–Sal fragment of xynA</td>
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</tr>
</tbody>
</table>

digested with EndoR and BglII. Electrophoresis was done in 1:2% agarose gels (SeaKem). The amplified DNA product was isolated 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 Luria broth medium containing ampicillin (100 μg/ml) 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, and concentrated by ultrafiltration (Amicon). The sample was applied to a column containing CM-Sepharose CL-6B (Pharmacia) and eluted with a linear gradient of 0-400 mM NaCl. Fractions containing lichenase were further purified by gel chromatography 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.

Xylanase was prepared from the supernatant by precipitating with ammonium sulfate (60% saturation) for 24 h at 4°C. The precipitate was resuspended and ultrafiltered twice against 0.1 M sodium acetate buffer, pH 5.0. FPLC chromatography using Sephacryl S-200 and Sephacryl S-200 was performed as described for β-glucanases.
of PBS1 grown on MW10 was used to infect the reference strains 1A6 (ide-A1 pyrD1 thyA1 thyB1 trpC2) and 1A7 (gltA292 trpC2; Dedonder et al., 1977). Transductants were scored for their ability to hydrolyse CM-cellulose. xynA 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 xynA, 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 eglS and bglS were detected after washing twice in 50% (v/v) formamide, 0.1% SDS, 36 M NaCl, 20 mM sodium phosphate, 2 mM EDTA for 20 min at 42 °C and once in 18 M 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 hydrolysing CM-cellulose were isolated from a λ47.1 library. A 5.6 kb HindIII DNA fragment isolated from λ47.1-Liec1 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 (Borriess 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 C120 (Murphy et al., 1984).

To construct an integration plasmid carrying a defective bglS gene, a 1.86 kb EcoRI–SphI 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 (ΔbglS555), was selected for further studies. Another deletion (ΔbglS’/bglSRV) within the 2-8 kb HindIII/SphI fragment was obtained by removing the 1-2 kb EcoRV fragment containing the 3’ part of bglS 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 λ47.1 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 pRB36 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 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 isoforms 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. 1b, 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 Vmax 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 translation termination signal formed by an inverted repeat of 14 nt at position 1095 [ΔG = −18.2 kcal (−76.1 kJ)].

To construct an integration plasmid carrying a defective eglS gene, we followed the strategy described for bglS. A DNA fragment containing 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 xynA gene

A 770 bp DNA fragment harbouring the entire xynA 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
Table 2. Substrate specificities of endo-1,4-β-glucanase (Egl, 1,3-1,4-β-glucanase (Bgl) and 1,4-β-xylanase (XynA) purified to electrophoretic homogeneity from E. coli cells harbouring recombinant plasmids pRB33 (bglS), pRB37 (egls) and pMW7 (xynA)

Enzyme activities were calculated from the results of three independent measurements.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Bgl (Units)</th>
<th>Egl (Units)</th>
<th>XynA (Units)</th>
</tr>
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<tbody>
<tr>
<td>Lichenan</td>
<td>2600*</td>
<td>368*</td>
<td>NM</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>NM</td>
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<td>NM</td>
</tr>
<tr>
<td>Birch wood xylan</td>
<td>NM</td>
<td>228*</td>
<td>NM</td>
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<tr>
<td>AZCL β-glucan</td>
<td>100†</td>
<td>5†</td>
<td>NM</td>
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<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>
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</tr>
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NM, not measurable.
*Unit defined as 1 μmol reducing sugar min⁻¹ (mg purified enzyme)⁻¹.
†Unit defined as 1 OD₅₅₀ unit min⁻¹ (mg purified enzyme)⁻¹.

Fig. 1. Restriction map and deletion analysis of bgl and egl. (a) Restriction map of a 5·6 kb HindIII fragment harbouring bglT and bglS. The ORFs of bglT and bglS are indicated by arrows. DNA fragments used to replace the chromosomal bgl genes are shown as filled boxes. The deleted regions Δbgl555 and Δbgl55R are indicated by parentheses. +, detection of lichenase activity on lichenan agar plates. Plasmids pMW3 and pMW4 were used to delete chromosomal bgl genes in B. subtilis DB104. Sequence analysis of Δegl555 revealed that 669 nt out of a total of 726 nt within the region encoding bglS have been deleted. (b) Restriction map of B. subtilis DNA harbouring an incomplete orfX (1–1074) and eglS (1380–2876). The map was constructed on the basis of two overlapping DNA fragments cloned in plasmids pAG1 and pRB36. The sequenced region and the length of the ORFs are indicated. The terminator-like sequence is labelled with a T. +, detection of cellulase activity on CM-cellulose agar plates. Plasmid pMW2 harbouring deletion Δegl5102 was used to replace the eglS gene. Sequence analysis of Δegl5102 revealed that 643 nt within the eglS region encoding E₁₅₀ to D₁₈₀ have been deleted.

Construction of mutants without β-glucanase and xylanase activity

Transformation of DB104 with integrating plasmid pMW5 carrying the deleted eglS gene with the flankng sequences (eglS102) 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 bgIS gene yielded only CmR BglIS⁻ clones. The BglIS⁻ phenotype was not detected. This result might be due to the size of the 1·2 kb deletion introduced in the bgIS region. Clones with the CmS BglIS⁻ phenotype were obtained after cultivation in the absence of antibiotic selection and one of them, named

...et al., 1986) except for a single substitution of T₁₈₄ to C₁₈₄. The resulting amino acid substitution, Ser₂₉₉ to Pro₂₉₉, is located within the signal peptide and does not affect the sequence of the mature protein. The apparent molecular mass of 20·5 kDa as calculated from the sequence and estimated by SDS-PAGE of the xylanase enzyme expressed in E. coli cells, agrees with the value reported for the PAP115 xylanase (data not shown). The purified product of xynA was active against oat spelt xylan and birch wood xylan but no activity against laminarin, lichenan or CM-cellulose could be detected (Table 2).
MW10, was chosen for further studies. Another deletion within the bgI5 gene was introduced using integrative plasmid pMW3. The Cr BglI phenotype was not observed after transformation into MW8. However, after cultivation without selection pressure, stable Cr BglI clones could be isolated. One of these was designated MW9. Southern hybridization verified that the transformed strains harbour the deleted bgI5 and egl5 genes (Fig. 2).

Another strategy was used to construct strains deficient in xylanase activity. The central 382 bp PstI-SacI 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). Cr 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 congenection into strain MW10 (EglI BglI). The resulting strains MW11–MW13 would exhibit a blue phenotype on X-Gal agar plates if transformed by plasmid pHPS9 (lacZat), 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 Cr 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 (EglI), did not hydrolyse CM-cellulose agar but was able to degrade lichenan. Double mutants harbouring AeglS 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 egl5 gene product is able to hydrolyse mixed linked β-glucans (Fig. 3). MW15 harbouring AeglS, AbglS and AxjnA was not able to hydrolyse lichenan, CM-cellulose or AZCL xylan. All strains were capable of

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Fig. 2. Southern blot analysis of chromosomal DNAs from DB104 and strains harbouring ΔbgI and Δegl genes. (a) HindIII/SphI digest of chromosomal DNAs hybridized with the 0·8 kb EcoRV-SphI fragment from pRB33 (bgI). Lanes: 1, MW9 with ΔbgI55; 2, MW10 with ΔbgI/SR5; 3, DB104 with the entire bgI gene. The positions of size standards (EcoRI/HindIII-digested λ) are indicated. Deletion ΔbgI55 (MW9) is 669 bp, and ΔbgI/SR5 is 1195 bp. (b) Chromosomal DNAs digested with PvuII and hybridized with the left-hand 1645 bp HindIII/EcoRI fragment from pRB36 (eglI). Lanes: 1, MW10 with ΔeglIS102; 2, DB104 with the entire eglI gene. The positions of size standards (ΔEcoRI/HindIII) are indicated. The 3· kb fragment visible in both lanes, indicates a 3· PvuII fragment harbouring orfX and the upstream sequence of eglI. (c) EcoRI-digested DNAs from _B. subtilis_ 168 (lane 1), YAC 12-5 (lane 2), YAC 15-37 (lane 3) and YAC 15-19 (lane 4) were probed with the amplified 760 bp xynA fragment. Note: this picture has been electronically imaged.
Fig. 3. Production of endoglucanases from mutant and wild type B. subtilis 168. Relevant genotypes of mutant strains: $\Delta$eglS102 (MW8), $\Delta$eglS102 AbglS55 (MW9), $\Delta$eglS102 Abgl bg/s5 (MW10) and $\Delta$eglS55 (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 $\beta$-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 ($\Delta$eglS); cellulase isoenzymes with an apparent molecular mass of 34–36 kb were detected in DB104 and MW14 ($\Delta$bg/s). 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.

bg/s, eglS and xynA map in different regions of the B. subtilis chromosome

With lysates prepared from 1A645 (zig85::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 ($\Delta$eglS, $\Delta$bg/s), and the resulting recombinants were checked for linkage to the eglS locus. The results shown in Fig. 5 demonstrate linkage of eglS to t/yA (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 ($\Delta$egl $\Delta$bg/s $\Delta$xynA) as recipient. Of the primary Em$^\text{R}$ transductants, 27% exhibited the XynA$^+$ phenotype as checked by plating on AZCL xylan agar. Dedonder kit strains 1A6 ($\text{thy}A^+$) and 1A7 ($\text{glt}A^+$) 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 $\text{thy}A^+--\text{glt}A^+$ 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

Table 3. Characterization of parental and mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Lichenan</th>
<th>CM-cellulose</th>
<th>Xylan</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB104</td>
<td>Wild type</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MW8</td>
<td>$\Delta$eglS02</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MW9</td>
<td>$\Delta$eglS02 $\Delta$bg/s55</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MW10</td>
<td>$\Delta$eglS02 $\Delta$bg/t bg/sR V</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MW11</td>
<td>$\Delta$eglS02 $\Delta$bg/t bg/sR V</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MW12</td>
<td>$\Delta$eglS02 $\Delta$bg/t bg/sR V</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MW13</td>
<td>$\Delta$eglS02 $\Delta$bg/t bg/sR V</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MW14</td>
<td>$\Delta$bg/s55</td>
<td>(+)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MW15</td>
<td>$\Delta$eglS02 $\Delta$bg/t bg/sR V $\Delta$xynA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Strains were streaked onto LB agar plates containing different polysaccharides and grown for 2 d. Hydrolysis of substrates is indicated by +. All strains degraded AZCL galactomannan but did not degrade AZCL pachyman or AZCL xyloglucan.
of the B. subtilis chromosome. The bglS 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 bglA 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, 1994).

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 gita 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 gita (177°) which is covered by YAC contigs 12-5, 15-37 and 15-19. The map shows the gene order thyA−egls−zei82−xynA−gita (Fig. 5). xynA, which is located at about 175°, is clearly separated from egls (170°) and the xyl−xynB cluster, which has been mapped at 168° on the B. subtilis chromosome (Azevedo et al., 1993).

**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 (bglS), 1,4-β-glucanase (egls) and 1,4-β-xylanase (xynA) have been cloned previously from different B. subtilis strains, especially from industrial strain PAP115, and structurally characterized. We cloned the bglS, egls and xynA genes from B. subtilis 168. The sequences of the egls and xynA genes from strain 168 were found to be virtually identical with the corresponding data reported for B. subtilis PAP115 (MacKay et al., 1986; Paice et al., 1986), suggesting that genes encoding extracellular enzymes are well conserved within B. subtilis strains.
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 (Yuuuki et al., 1989). According to its primary sequence, the gene belongs to family 16 of the glycosyl hydrolases formed by bacterial endo-1,3-1,4-/?-glucanases (Henrissat & Bairoch, 1993). The substrate specificity of endo-1,3-1,4-/?-glucanase is restricted to mixed linked ?-glucans such as lichenan or barley ?-glucan. No other hemicellulosic or cellulotic substrates are hydrolysed (Table 2).

The B. subtilis egls gene product (endo-1,4-/?-glucanase) has been classified into family 5 of the glycosyl hydrolases (Henrissat, 1991). The enzyme is highly similar to other bacterial endo-1,4-/?-glucanases and is able to hydrolyse CM-cellulose and mixed linked ?-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-/?-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-/?-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 bgls genes responsible for about 5–10% of total 1,3-1,4-/?-glucan hydrolising 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-/?-glucanase degrades mixed linked ?-glucans much faster than the 1,4-/?-glucanase (see above). The activity of lichenanase, Bgls, was found to be seven times higher on lichenan and about 20 times higher on AZCL ?-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 ?-glucans.

Strain MW15 (Δegls 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-/?-glucan and xylan hydrolysis in B. subtilis.

The B. subtilis 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-/?-glucan hydrolases are very distant from each other on the B. subtilis chromosome. The egls gene was genetically mapped close to the thyA 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 cirB gene region. Similarly, the genetic position of bgls determined by PBS1-mediated transduction at about 340°, adjacent to hut locus, was verified by hybridization of bgls DNA with YAC 13-2 containing the hut operon.

The same combined genetic and physical approach has proven useful in mapping the xynA gene. Phage PBS1-mediated transduction with strains harbouring deletions within xynA and the ?-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 gusB.

A gene cluster with xyl and xynC–xynB genes was located near thyA at 168° (Hastrup, 1988). The xyl gene was also physically mapped on YAC 12-5 harbouring the thyA 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 thyA 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 ?-glucan and xylan degradation in B. subtilis. The mutants deficient in endo-/?-glucanases and xylanase described here may well prove useful for secretion of heterologous ?-glucan hydrolases and xylanases. In addition, direct cloning of genes encoding ?-glucan hydrolases might be facilitated by using the a-complementation system introduced into strain MW12.

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