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

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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 -1,3-1,4-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 cellulosic 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. pumilus* 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 (*bgl*), Z29076 (*egl*) and Z34519 (*xynA1*).

the *sacA-purA* region of the *B. subtilis* chromosome and is closely linked to the *hut* locus (O'Kane *et al.*, 1985; Borriss *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 (Borriss *et al.*, 1986).

xynA and *xynB* mutants (deficient in extracellular xylanase and cell-bound β -xylosidase, respectively) unable to use xylan as sole carbon source were mapped at 48° 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* 168 (Hastrup, 1988). Azevedo *et al.* (1993) mapped *xyl* 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 near *thyA* (168°) and *citB* (173°). 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 cellulase are not clustered but rather map at different sites on the chromosome. *Egl*[−] *Bgl*[−] 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 °C, 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% of the substrate to LB agar. Extracellular enzyme activity was indicated by liquefaction 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 °C, 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 $V_{\max}$ 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 (2.0%, w/v), CM-cellulose (0.5%), oat spelt and birch wood xylan (0.5%) were used as substrates. One unit of enzyme activity is defined as the amount of enzyme producing 1 μ mol 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 spelts as substrate. AZCL polysaccharide (5 mg) in 20 mM sodium acetate buffer, pH 6.0, supplemented with 10 mM CaCl₂ and 50 μ g 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 OD₅₉₅ of 1.0 min^{−1}.

Detection of β -galactosidase. When β -galactosidase α -complementation was assayed, either X-Gal (60 μ g 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 λ L47.1 (Putzer *et al.*, 1990). DNA from phage clones exhibited β -glucan hydrolysing activity was isolated, digested by *Hind*III and subcloned into vector pBR322 (Bolivar *et al.*, 1977). Clones hydrolysing CM-cellulose (*eglS*) and lichenan (*bglS*) were selected. Isolated plasmids were shown to direct expression of *eglS* (pRB36) and *bglS* (pRB31). Another approach to isolating genes encoding β -glucan hydrolases used vector plasmid pBR322. Chromosomal DNA isolated from *B. subtilis* 168 was partially digested by *Sau*III and size-fractionated by agarose gel electrophoresis. DNA fragments of 2–10 kb in size were ligated into pBR322 linearized by *Bam*HI and dephosphorylated by alkaline phosphatase. *E. coli* DH5 α 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 dideoxy-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 μ l contained 100 ng *Pvu*II-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 nucleoside triphosphate (NTP) and 2 U *Taq* polymerase. The sequences of the primers were deduced from the published sequence of the *B. subtilis* PAP115 *xynA* gene (Lindner *et al.*, 1994): *xynA*/oli1, 5' CTGAATTCGTGGTATTACTGAAGG; *xynA*/oli2, 5' CCTGATTAAGGAAGATCTGTTACC. The restriction sites for *Eco*RI (*xynA*/oli1) and *Bgl*/II (*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

Table 1. Strains and plasmids

Strain/plasmid	Characteristics	Source/reference
B. subtilis		
168	<i>trpC2</i>	BGSC, Ohio, USA
6GM15	<i>trpC2 tyr met his ura rib</i> ($r_m^-m_m^+$) <i>lacZ</i> ΔM15 Km ^R	Haima <i>et al.</i> (1990)
DB104	<i>his nprR2 nprE18 ΔaprA3</i>	Kawamura & Doi (1984)
MW8	<i>his nprR2 nprE18 ΔaprA3 ΔeglS102</i>	This work
MW9	<i>his nprR2 nprE18 ΔaprA3 ΔeglS102 ΔbglS55</i>	This work
MW10	<i>his nprR2 nprE18 ΔaprA3 ΔeglS102 ΔbglT bglSRV</i>	This work
MW11	<i>his nprR2 nprE18 ΔaprA3 ΔeglS102 ΔbglT bglSRV ura rib</i> ($r_m^-m_m^+$) <i>lacZ</i> ΔM15 Km ^R	This work
MW12	<i>his nprR2 nprE18 ΔaprA3 ΔeglS102 ΔbglT bglSRV trpC2 ura rib</i> ($r_m^-m_m^+$) <i>lacZ</i> ΔM15 Km ^R	This work
MW13	<i>his nprR2 nprE18 ΔaprA3 ΔeglS102 ΔbglT bglSRV trpC2 tyr ura rib</i> ($r_m^-m_m^+$) <i>lacZ</i> ΔM15 Km ^R	This work
MW14	<i>his nprR2 nprE18 ΔaprA3 ΔbglS55</i>	This work
MW15	<i>his nprR2 nprE18 ΔaprA3 ΔeglS102 ΔbglT bglSRV ΔxynA</i> Cm ^R	This work
YACs		
YAC 12-5	130 kb YAC insert with <i>thyA xyl citB</i> (map position 165°)	P. Serron, INRA, Jouy-en-Josas, France
YAC 15-37	130 kb YAC insert with <i>citB</i> (map position 169°)	
YAC 15-19	175 kb YAC insert with <i>gltA terC odhA</i> (map position 173°)	
Plasmid		
pHPS9	Cm ^R Er ^R , <i>cat-86::lacZ</i> α, pTA1060-pUC9 derivative	Haima <i>et al.</i> (1990)
pRB31	pBR322 containing a 5·6 kb <i>Hind</i> III fragment of <i>bgl</i> of <i>B. subtilis</i>	This work
pRB33	pBR322 containing a 2·8 kb <i>Hind</i> III- <i>Sph</i> I fragment of <i>bglS</i>	This work
pAG1	pBR322 containing a 3·6 kb fragment of <i>egl</i> of <i>B. subtilis</i>	This work
pAG2	pBR322 containing a 1·9 kb fragment of <i>egl</i> of <i>B. subtilis</i>	This work
pRB36	pBR322 containing a 2·8 kb <i>Hind</i> III of <i>egl</i> of <i>B. subtilis</i>	This work
pRB37	pUC19 containing a 2·8 kb <i>Hind</i> III of <i>egl</i> of <i>B. subtilis</i>	This work
pHV60	Ap ^R Tc ^R Cm ^R , pBR322 derivative, <i>cat</i> gene from pC194	Michel <i>et al.</i> (1983)
pMW1	pHV60 containing a 2·8 kb <i>Hind</i> III- <i>Sph</i> I fragment of pRB33	This work
pMW2	pHV60 containing a 4·3 kb <i>Eco</i> RV- <i>Sal</i> I fragment of pAG1	This work
pMW3	pMW1 containing a 0·7 kb deletion within <i>bglS</i>	This work
pMW4	pMW1 containing a 1·2 kb deletion within the <i>bglT-bglS</i> region	This work
pMW5	pMW2 containing a 0·7 kb deletion within <i>eglS</i>	This work
pMW6	pBR322 containing 770 bp of <i>xynA</i>	This work
pMW7	pTZ18R containing 770 bp of <i>xynA</i>	This work
pMW8	pHV60 containing an internal <i>Pst</i> I- <i>Sca</i> I fragment of <i>xynA</i>	This work

digested with *Eco*RI and *Bgl*II. 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 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 5.0, 10 mM CaCl₂, 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. FPLC chromatography with CM-Sepharose and Sephacryl S-200 was performed as described for β -glucanases.

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 (Borriess *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. Em^R transductants were scored for their ability to hydrolyse lichenan and CM-cellulose. To map the *eglS* gene more precisely, a lysate

of PBS1 grown on MW10 was used to infect the reference strains 1A6 (*ilvA1 pyrD1 thyA1 thyB1 trpC2*) and 1A7 (*gluA292 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 *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 & Borriess, 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 λ 47.1 library. A 5.6 kb *Hind*III DNA fragment isolated from λ 47.1-Lic1 directs lichenan-degrading activity. The fragment was cloned into pBR322. *E. coli* DH5 α 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 *Eco*RI-*Sph*I fragment carrying the entire sequence of *bglS* was blunt-ended and cloned into the unique *Eco*RV site of vector pHV60. The resultant plasmid, pMW1, was linearized with *Eco*RV and treated with *Bal*31 exonuclease. Deletions of 400–900 bp were obtained. After religation, plasmid pMW3, carrying a deletion within the *bglS* gene of about 700 bp (Δ *bglS*55), was selected for further studies. Another deletion (Δ *bglT/bglSRV*) within the 2.8 kb *Hind*III/*Sph*I fragment was obtained by removing the 1.2 kb *Eco*RV 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 λ 47.1 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 55 287 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 52 264 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 *Hind*III 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 [$\Delta G = -18.2$ 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 *Mlu*I, treated with *Bal*31 and religated. A clone with a 643 bp deletion within *eglS* (Δ *eglS*102) 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

Fig. 1. Restriction map and deletion analysis of *bgl* and *egl*. (a) Restriction map of a 5.6 kb *Hind*III 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 $\Delta bglS55$ and $\Delta bglSRV$ 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 $\Delta bglS55$ 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 $\Delta eglS102$ was used to replace the *eglS* gene. Sequence analysis of $\Delta eglS102$ revealed that 643 nt within the *eglS* region encoding E₁₆₉ to D₃₈₅ have been deleted.

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.

Substrate	Bgl (Units)	Egl (Units)	XynA (Units)
Lichenan	2600*	368*	NM
CM-cellulose	NM	45*	NM
Oat spelt xylan	NM	NM	316*
Birch wood xylan	NM	NM	228*
AZCL β -glucan	100†	5†	NM
AZCL HE-cellulose	NM	20†	NM
AZCL xylan	NM	0.05†	234†

NM, not measurable.

* Unit defined as 1 μ mol reducing sugar min^{-1} (mg purified enzyme) $^{-1}$.

† Unit defined as 1 OD₅₉₅ unit min^{-1} (mg purified enzyme) $^{-1}$.

et al., 1986) except for a single substitution of T₁₈₄ to C₁₈₄. The resulting amino acid substitution, Ser₂₃ to Pro₂₃, 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).

Construction of mutants without β -glucanase and xylanase activity

Transformation of DB104 with integrating plasmid pMW5 carrying the deleted *eglS* gene with the flanking sequences ($\Delta eglS102$) yielded transformants with two different phenotypes: most of the transformants were Cm^R EglS⁺ but about 10% were found to be Cm^R, 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, Cm^R EglS⁻ transformants were cultivated for about 10 generations in antibiotic-free medium. One cured Cm^S clone, designated MW8, with a stable EglS⁻ phenotype was selected.

Transformation of MW8 with the integrative plasmid pMW4 carrying the deleted *bglS* gene yielded only Cm^R 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 Cm^S BglS⁻ phenotype were obtained after cultivation in the absence of antibiotic selection and one of them, named

Fig. 2. Southern blot analysis of chromosomal DNAs from DB104 and strains harbouring Δbgl and Δegl genes. (a) *HindIII/SphI* digest of chromosomal DNAs hybridized with the 0.8 kb *EcoRV-SphI* fragment from pRB33 (*bgl*). Lanes: 1, MW9 with $\Delta bgl55$; 2, MW10 with $\Delta bglSRV$; 3, DB104 with the entire *bgl* gene. The positions of size standards (*EcoRI/HindIII*-digested λ) are indicated. Deletion $\Delta bgl55$ (MW9) is 669 bp, and $\Delta bglSRV$ is 1195 bp. (b) Chromosomal DNAs digested with *PvuII* and hybridized with the left-hand 1645 bp *HindIII/EcoRI* fragment from pRB36 (*egl*). Lanes: 1, MW10 with $\Delta eglS102$; 2, DB104 with the entire *egl* gene. The positions of size standards (λ *EcoRI/HindIII*) are indicated. The 3 kb fragment visible in both lanes, indicates a 3' *PvuII* fragment harbouring *orfX* and the upstream sequence of *eglS*. (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.

MW10, was chosen for further studies. Another deletion within the *bglS* gene was introduced using integrative plasmid pMW3. The Cm^R Bgl^- phenotype was not

observed after transformation into MW8. However, after cultivation without selection pressure, stable Cm^S 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-ScaI* 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). Cm^R 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 (*lacZ α*), 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 (Borriess *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 Cm^R 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 ($\Delta eglS$), did not hydrolyse CM-cellulose agar but was able to degrade lichenan. Double mutants harbouring $\Delta eglS$ and $\Delta bglS$ lacked 1,3-1,4- β -glucan hydrolysing activity, whereas strain MW14 with $\Delta bglS55$ 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 $\Delta eglS$, $\Delta bglS$ and $\Delta xynA$ 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: Δ *eglS102* (MW8), Δ *eglS102* Δ *bglS55* (MW9), Δ *eglS102*, Δ *bgl bglSRV* (MW10) and Δ *bglS55* (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 (Δ *eglS*); cellulase iso-enzymes with an apparent molecular mass of 34–36 kb were detected in DB104 and MW14 (Δ *bglS*). The apparent molecular mass of cellulase iso-enzymes 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 (*zif85*: :Tn917) containing a silent Tn917 insertion at 342°, 87.3% co-transduction 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 (Δ *eglS*, Δ *bglS*), 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 (Δ *egl* Δ *bgl* Δ *xynA*) as recipient. Of the primary Em^R transductants, 27% exhibited the XynA⁺ phenotype as checked by plating on AZCL xylan agar. Dedonder kit strains IA6 (*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

Table 3. Characterization of parental and mutant strains

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.

Strain	Relevant genotype	Lichenan	CM-cellulose	Xylan
DB104	Wild type	+	+	+
MW8	Δ <i>eglS102</i>	+	–	+
MW9	Δ <i>eglS102</i> Δ <i>bglS55</i>	–	–	+
MW10	Δ <i>eglS102</i> Δ <i>bglT bglSRV</i>	–	–	+
MW11	Δ <i>eglS102</i> Δ <i>bglT bglSRV</i>	–	–	+
MW12	Δ <i>eglS102</i> Δ <i>bglT bglSRV</i>	–	–	+
MW13	Δ <i>eglS102</i> Δ <i>bglT bglSRV</i>	–	–	+
MW14	Δ <i>bglS55</i>	(+)	+	+
MW15	Δ <i>eglS102</i> Δ <i>bglT bglSRV</i> Δ <i>xynA</i>	–	–	–

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. Apparent molecular masses are indicated. (a) SDS-PAGE gel with CM-cellulose. Lanes: 1, 0.5 µg purified 1,3-1,4-endo- β -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- β -glucanase. Cellulase isoenzymes with molecular masses ranging from 33 to 36 kDa are visualized in lane 7. Strains with the Δ *egl102* deletion (MW8, MW9 and MW10) were without detectable cellulase activity; purified lichenase (lane 1) did not degrade CM-cellulose. (b) SDS-PAGE gel with lichenan. Lanes were loaded as indicated in (a). Note that the supernatant of DB104 (lane 2) contains two lichenan hydrolysing activities with apparent molecular masses of 24 kDa (lichenase) and 33 kDa (cellulase). Lichenan is hydrolysed by purified 1,3-1,4-endo- β -glucanase (1) and 1,4-endo- β -glucanase (7). Strains harbouring either the intact *bgl* gene (MW8) or the intact *egl* gene (MW14) are able to degrade mixed linked β -glucan. Note: this picture has been electronically imaged.

of the *B. subtilis* chromosome. The *bglS* gene hybridized with a 190 kb fragment carrying the *hut* 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 *hut* (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 *Eco*RI-digested DNA prepared from dif-

Fig. 5. Linkage relationships of *thyA*, *eglS*, *zei82*::Tn917, *xynA* and *gltA* 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*-*gltA* 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 & Borris, 1995).

ferent 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*. *Eco*RI-digested DNA prepared from YAC 15-19 carrying *gltA* 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 *gltA* (177°) which is covered by YAC contigs 12-5, 15-37 and 15-19. The map shows the gene order *thyA*-*eglS*-*zei82*-*xynA*-*gltA* (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 (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 cellulose substrates are hydrolysed (Table 2).

The *B. subtilis* *eglS* gene product (endo-1,4- β -glucanase) has been classified into family 5 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 (Δ *eglS* Δ *bglS*) were without detectable activity towards lichenan and CM-cellulose, suggesting that the minor β -glucanases responsible for about 5–10% of total 1,3-1,4- β -glucan hydrolysing activity found in *bglS* mutant strains of *B. subtilis* 168 (Borriess *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 lichenase, 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 (Δ *bglS* Δ *eglS* Δ *xynA*), 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 *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- β -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 *citB* 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 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 *guaB*.

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 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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