Mapping and Properties of *bgl* (β -Glucanase) Mutants of *Bacillus subtilis*

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(Received 4 July 1985; revised 19 September 1985)

Among secreted proteins of *Bacillus subtilis* 168 four polypeptides, separated by electrophoresis in polyacrylamide gradient gels under denaturing conditions, were shown to have lichenan-hydrolysing enzyme activity; one of them, a 22 kDa protein, represents the main β -glucanase activity. A number of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced mutants defective in β -glucanase activity at an elevated temperature (44 °C) were isolated and characterized. All mutants analysed by PAGE had no active 22 kDa β -glucanase in their supernatants and showed no cross-reactivity with antibodies raised against purified 22 kDa β -glucanase. However, formation of other exoenzymes, including the other minor β -glucanases, was not affected in these mutant strains. The mutations (*bgl-1*, *bgl-12*, *bgl-35*, *bgl-101*) were mapped by phage PBS1-mediated transduction and found to be located between *purA* and *sacA*, close to the *hutH* marker of the *B. subtilis* chromosome. A 3.8 kb *EcoRI* fragment of the *B. subtilis* chromosome which directs β -glucanase synthesis in *Escherichia coli* as well as complementing a β -glucanase deficient mutant, *bgl-35*, of *B. subtilis* to synthesize active 22 kDa β -glucanase was mapped after homologous recombination by means of an integratable plasmid. The cointegrated chloramphenicol-resistance marker of the plasmid was found at the same map position as the mutations *bgl-1*, *bgl-12*, *bgl-35* and *bgl-101*, suggesting that the gene affected encodes for the 22 kDa β -glucanase and lies within the order *ctrA-sacA-bgl-purA*.

INTRODUCTION

Enzymes which hydrolyse mixed β -glucans containing β -1,3 and β -1,4 linkages are present in the culture fluid of several *Bacillus* species (Borriss *et al.*, 1980). The substrate range of these bacterial β -glucan-endohydrolases is similar to lichenase (EC 3.2.1.73; 1,3-1,4- β -D-glucan 4-glucanohydrolase) of germinating barley and is restricted to mixed-linked glucans like lichenan and barley β -glucan (Anderson & Stone, 1975). The purified enzymes of *B. subtilis* (Moscatelli *et al.*, 1961), *B. pumilus* (Suzuki & Kaneko, 1976) and *Bacillus* sp. IMET B376 (Borriss, 1981) are more completely characterized. The molecular masses of these extracellular enzymes are estimated to be in the range 25 to 30 kDa.

We have described the formation of the enzyme during growth in batch (Borriss, 1976) and continuous (Borriss *et al.*, 1982) culture, its substrate specificity and action patterns (Borriss & Zemek, 1980), its biochemical properties (Borriss & Zemek, 1981) and the beneficial effect on wort viscosity caused by hydrolysis of high polymer barley β -glucans during the brewing process (Borriss & Schroeder, 1981). However, unlike other extracellular enzymes, the β -glucanase producing system of *Bacillus* has not been studied genetically until recently.

This paper deals with the characterization and mapping of various mutants of *B. subtilis* altered in β -glucanase formation (*bgl* mutants). Furthermore, we describe cloning of a *B. subtilis* 168 chromosomal fragment expressing β -glucanase in *B. subtilis*. After chromosomal insertion of

Abbreviation: NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

the cloned fragment the ability of the *bgl-35* mutant to synthesize the 22 kDa β -glucanase was fully restored.

METHODS

Bacterial strains. All strains used in this study are listed in Table 1.

Media and growth conditions. Strains were routinely grown in TBY (10 g Difco tryptone, 5 g yeast extract, 5 g NaCl per litre) or NBY (10 g peptone, 5 g yeast extract, 5 g NaCl per litre) at 37 °C under aeration by vigorous shaking (220 r.p.m.). Antibiotics, when necessary, were added at the following concentrations: tetracycline (Tc), 20 $\mu\text{g ml}^{-1}$; chloramphenicol (Cm), 5 $\mu\text{g ml}^{-1}$; ampicillin (Ap), 30 $\mu\text{g ml}^{-1}$. Solid media were made with 2% (w/v) agar. When appropriate, TBY plates were supplemented with 0.2% lichenan.

Isolation of mutants. This was done according to the procedure of Balassa (1969). Germinating spores were incubated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (0.5 mg ml^{-1}) for 60 min at 37 °C in 0.1 M-sodium acetate buffer pH 6. Immediately after mutagenesis and washing, samples of cells were stored in separate tubes with 20% (v/v) glycerol at -20 °C. Mutants affected in the production of extracellular β -glucanase were isolated by plating mutagenized cultures onto nutrient agar plates containing 1% (w/v) lichenan (Borriss *et al.*, 1980). After incubation for 20 h at 44 °C colonies without haloes in turbid lichenan agar were picked and purified at least once by streaking onto the same agar.

Transformation and transduction. Chromosomal DNA was prepared according to Dubnau & Davidoff-Abelson (1971). Competent cells were prepared according to the method of Bott & Wilson (1967) in a minimal medium (Bott & Wilson, 1968), supplemented with auxotrophic requirements at 50 $\mu\text{g ml}^{-1}$. Transformation was done by adding DNA to 1 ml of a competent culture to give a saturating concentration of 1 $\mu\text{g ml}^{-1}$ for strain construction and 0.02 $\mu\text{g ml}^{-1}$ for mapping experiments, respectively. The cells were exposed to DNA for 30 min and plated for selection on minimal medium (Anagnostopoulos & Spizizen, 1961) supplemented with auxotrophic requirements to 20 $\mu\text{g ml}^{-1}$, except for thymine (50 $\mu\text{g ml}^{-1}$) and glutamate or aspartate (50 $\mu\text{g ml}^{-1}$). Recombinants capable of growth on histidine as the sole carbon and nitrogen source were selected on minimal histidine plates with 0.2% histidine and 0.001% yeast extract (Chasin & Magasanik, 1968).

Plasmids were transformed in *B. subtilis* by the protoplast transformation method (Chang & Cohen, 1979) and in *Escherichia coli* according to Kushner (1978).

Phage PBS1-mediated transduction was done as described by Love *et al.* (1976), except that the chloroform and DNAase treatments were omitted. Transduction was effected by mixing 1 vol. donor lysate (0.1 to 2.0 $\times 10^9$ p.f.u. ml^{-1}) with 10 vols recipient culture in the late exponential phase of growth. Mobility of cells was checked by phase contrast microscopy. The infected cells were plated for selection on minimal agar plates as described for transformation.

Detection of extracellular enzyme formation. β -Glucanase was detected on agar plates as described previously (Borriss *et al.*, 1985). β -Glucanase activity was assayed by determination of liberated reducing sugars from lichenan by the dinitrosalicylic acid method (Borriss, 1981).

α -Amylase was measured by using SPOFA TEST tablets (SPOFA, Prague, Czechoslovakia) according to the specifications given by the supplier.

Protease was determined, using azocasein as substrate, according to Langner *et al.* (1973).

All enzyme activities were expressed in optical units $\text{ml}^{-1} \text{h}^{-1}$.

Sporulation. Strains were grown in double-strength NBY supplemented with $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.25 g l^{-1}), KCl (1 g l^{-1}), MnCl_2 (10 mM), FeSO_4 (1 mM), $\text{Ca}(\text{NO}_3)_2$ (1 mM). Sporulation frequencies were determined by the ratio of the number of heat resistant spores (surviving heating to 80 °C for 10 min) and to the number of cells present after 2 d growth in sporulation medium. Both types of cells were counted as colony formers after plating on nutrient agar.

Susceptibility to phage PZA. This was tested according to Fučík *et al.* (1982).

PAGE and detection of β -glucanase. The strains were cultured for 24 h as described above. The protein from 1.5 ml growth supernatant was precipitated with 10% (w/v) TCA. Samples containing about 0.5 mg protein were dissolved in 200 μl of 50 mM-sodium borate pH 8.9, 8 M-urea, 2% (w/v) SDS, 10 mM-dithiothreitol, 1 mM-*p*-aminocaproic acid and 1 mM-phenylmethylsulphonyl fluoride, heated for 3 min in a boiling water bath and centrifuged at 20000 *g* for 5 min. Protein extracts (20 μg protein) were electrophoretically separated on 8–18% (w/v) polyacrylamide gradient slab gels containing 0.1% SDS and 5 M-urea (Süss & Schmidt, 1982). To detect β -glucanase activities, the gel was washed three times each for 1 h with 200 ml 50% (v/v) ethanol in order to remove urea and SDS. The gel was reswollen in 0.02 M-sodium acetate buffer pH 6 for 1 h and then laid onto a 10% polyacrylamide gel containing 0.3% lichenan and kept for 20 h at 25 °C. Afterwards the gradient gel was stained for protein with Coomassie brilliant blue G250 and the lichenan-containing gel was immersed in 0.1% Congo red for 10 min to visualize β -glucanase activity. Enzyme bands appeared as white zones on a red background.

Serology. Polyclonal antiserum against β -glucanase was produced in rabbits by injection of a purified β -glucanase preparation isolated from *B. subtilis* (Borriss *et al.*, 1985). The rabbit was bled 5 weeks after injection.

Table 1. *Strains, phages and plasmids used*

Genotype or relevant phenotype and properties*		Origin/reference†
<i>B. subtilis</i> strains		
168	<i>trpC2</i>	R. Dedonder
QB944 (kit1)	<i>purA16 cycA14 trpC2</i>	
QB928 (kit2)	<i>aro1906 purB33 trpC2</i>	
QB934 (kit3)	<i>tre-12 metC3 glyB133 trpC2</i>	
QB943 (kit4)	<i>pyrD1 ilvA1 thyB1 trpC2</i>	
QB922 (kit5)	<i>gltA292 trpC2</i>	
QB935 (kit6)	<i>aroD120 lys-1 trpC2</i>	
QB936 (kit7)	<i>leuA8 aroG932 ald-1 trpC2</i>	
QB917 (kit8)	<i>hisA1 thr-5 trpC2</i>	
QB123 (kit9)	<i>sacA321 ctrA1 trpC2</i>	BGSC
1A272	<i>hutH1</i>	
BG301	<i>bgl-1 trpC2</i>	NTG on strain 168
BG302	<i>bgl-12 trpC2</i>	
BG303	<i>bgl-26 trpC2</i>	
BG304	<i>bgl-35 trpC2</i>	
BG307	<i>bgl-101 trpC2</i>	
BG308	<i>bgl-102 trpC2</i>	
BG309	<i>bgl-35 purA16 trpC2</i>	BG304 $\xleftrightarrow{u}$ QB944
BG310	<i>bgl-102 sacA321 trpC2</i>	BG308 $\xleftrightarrow{u}$ QB123
BG311	<i>bgl-1 aroD120 trpC2</i>	BG301 $\xleftrightarrow{u}$ QB935
BG312	<i>bgl-12 aroD120 trpC2</i>	BG302 $\xleftrightarrow{u}$ QB935
BG313	<i>bgl-26 aroD120 trpC2</i>	BG303 $\xleftrightarrow{u}$ QB935
BG314	<i>bgl-35 aroD120 trpC2</i>	BG304 $\xleftrightarrow{u}$ QB935
BG315	<i>bgl-101 aroD120 trpC2</i>	BG307 $\xleftrightarrow{u}$ QB935
BG317	<i>purA16 bgl-35 trpC2</i>	BG304 $\xleftrightarrow{u}$ QB944
BG318	<i>sacA321 bgl-102 trpC2</i>	BG308 $\xleftrightarrow{u}$ QB123
BG319	<i>bgl-35 argA15 Res⁻ Mod⁻</i>	BG 304 $\xleftrightarrow{u}$ RM125
BG320	<i>Cm^r argA15 Res⁻ Mod⁻</i>	pHV60-28 $\xleftrightarrow{u}$ BG319
RM125	<i>leuA8 argA15 Res⁻ Mod⁻</i>	V. Fučík
RM125/2	<i>recE4 leuA8</i>	V. Fučík
<i>E. coli</i> strains		
HB101	<i>HsdS⁻ Leu⁻ Pro⁻ Lac⁻ Str^r thi-1 recA1</i>	Boyer & Roulland-Dussoix (1969)
DP50	<i>supF</i> , host strain for Charon 4A	Blattner <i>et al.</i> (1977)
Plasmids		
pBR322	Ap ^r Tc ^r	Bolivar <i>et al.</i> (1977)
pHV60	Ap ^r Tc ^r Cm ^r	Michel <i>et al.</i> (1983); S. D. Ehrlich
pHV60-28	Ap ^r Tc ^r Cm ^r Bgl ⁺	This study
pHV33	Ap ^r Tc ^r Cm ^r	Primrose & Ehrlich (1981)
Phages		
PBS1	<i>B. subtilis</i> transducing phage	Takahashi (1963)
PZA	<i>B. amyloliquefaciens</i> phage	Fučík <i>et al.</i> (1982)
Charon 4A	<i>E. coli</i> replacement vector phage	Blattner <i>et al.</i> (1977)

* *bgl*, Mutation in β -glucanase formation.

† tf, Transformed by DNA isolated from the donor strain; R. Dedonder, CNRS et Université Paris VII, place Jussieu, France (Dedonder *et al.*, 1977); V. Fučík, Institute of Molecular Genetics, Flemingovo nám. 2, 166 10 Praha 6, Czechoslovakia; BGSC, Bacillus Genetic Stock Center, Ohio State University, Columbus, Ohio 43210, USA; S. D. Ehrlich, Institute de Recherche en Biologie Moléculaire, 2 Place Jussieu, Paris, France.

The activity of the antiserum was tested by immunotitration of enzyme activity with increasing volumes of antiserum. The specificity of antiserum was tested by immunoblotting (Towbin *et al.*, 1979).

Proteins of *B. subtilis* culture fluids were separated on SDS/urea-polyacrylamide gradient slab gels as described above and transferred onto nitrocellulose filters. Antibody binding was done with a 1:20 dilution of antiserum in 10 mM-Tris pH 7.4, 0.9% NaCl, containing 3% (w/v) bovine serum albumin. The immune complex was detected with [¹²⁵I]protein A (10⁶ c.p.m. ml⁻¹) followed by autoradiography.

Cloning procedures. The isolation of chromosomal DNA from *B. subtilis* 168, preparation of phage DNA and plasmid DNA from *E. coli* cells, ligation and *in vitro* packaging were done as described previously (Borriess *et al.*, 1985). Plasmid DNA from *B. subtilis* was prepared as described by Gryczan *et al.* (1978).

Restriction endonucleases and 4 ligase. Enzymes used in this study, except *PvuI* (Boehringer-Mannheim), were from Zentralinstitut für Mikrobiologie und experimentelle Therapie, Jena, and used as recommended by the supplier.

Southern blotting. Transfer of DNA from gels onto nitrocellulose filters, and subsequent probing by labelled DNA, was done according to the method of Southern (1979).

RESULTS

Detection of lichenan-hydrolysing enzymes in B. subtilis

For detection of lichenan-hydrolysing enzymes we have developed a two-step technique consisting of electrophoretic separation of proteins from bacterial culture fluids under denaturing conditions followed by electrophoretic transfer of the separated and renatured proteins into a second acrylamide gel containing the enzyme substrate lichenan. Clearing zones caused by lichenan-hydrolysing enzymes become visible after staining with Congo red (see Methods).

Using this enzymographic technique, four protein bands were shown to possess lichenan-hydrolysing enzyme activity; among them the 22 kDa β -glucanase (β -glucanase I) seemed to represent the main β -glucanase activity of *B. subtilis* 168. Other minor lichenan-hydrolysing activities (β -glucanases II, III and IV) were associated with 33–36 kDa polypeptides. These polypeptides showed no cross-reactivity with antibodies raised against purified 22 kDa β -glucanase (Fig. 1).

Isolation of β -glucanase mutants

The procedure for isolating *B. subtilis* 168 mutants after NTG treatment is described in Methods. To detect mutants defective in or temperature-sensitive for β -glucanase activity, the screening procedure was done at 44 °C. From about 100 000 colonies tested, 138 showed decreased β -glucanase activity. Among these only six mutants showed no haloes during growth at 44 °C on lichenan agar, and these were kept for further investigations. The frequency of *bgl* mutants (defective in β -glucanase activity under these conditions) was in good agreement with a mutation rate of 4×10^{-6} as estimated for the reversion of the *trpC2* marker under identical conditions of mutagenesis.

Properties of selected mutants

Extracellular enzyme activities were determined after growth of mutant strains or QB935 (as a control) in double-strength NBY medium at 37 °C. β -Glucanase activity secreted by the mutant strains was less than 5–10% of that of the wild-type strain (Table 2). Applying the enzymographic technique to detect glucanase activity of renatured proteins separated by SDS-PAGE, it was clear that the mutant strains had lost active 22 kDa β -glucanase (β -glucanase I) but the other three minor 'lichenase' activities were still clearly visible (Fig. 1). To determine whether an inactive β -glucanase protein was secreted by *bgl* mutant strains, electrophoretically separated proteins of culture fluids were transferred to nitrocellulose and immunoblotted with antiserum against purified 22 kDa β -glucanase. There was no cross-reacting material in the mutant strains (Fig. 1).

The formation of other extracellular enzymes, including α -amylase and protease, was not affected in strains harbouring *bgl-1*, *bgl-12*, *bgl-35* and *bgl-101* mutations. However, the protease activity of *bgl-26* and *bgl-102* was somewhat reduced, and additionally in *bgl-26* α -amylase activity was strongly decreased (Table 2).

Mutants blocked in early stages of sporulation (*spo0*) might have lost some of the sporulation-associated functions, i.e. ability to form extracellular enzymes, especially proteases (Brehm *et al.*, 1983). To test the possibility that in some of our mutants the *bgl* phenotype was caused by a *spo0* mutation we assayed the efficiency of plating of *Bacillus amyloliquefaciens* phage PZA on *bgl* mutants. Phage PZA only forms plaques on asporogenous mutants blocked at early stages of sporulation (Fučík *et al.*, 1982). No plaques of PZA appeared on a lawn of *B. subtilis* QB935 or any of the *bgl* mutants even at very high multiplicities of infection (Table 2), indicating that the

Fig. 1. Proteins from culture fluids of *B. subtilis* *bgl* mutant (strain BG314) and wild-type (strain QB935). The proteins were separated on 8–18% polyacrylamide gradient gel and stained by Coomassie brilliant blue G250 (a), or blotted onto nitrocellulose strips prepared with antibodies against purified 22 kDa β -glucanase (b), or used for detection of β -glucanase activity by sandwiching the gel on a second polyacrylamide gel containing lichenan (c). Lane 1, strain BG314; lane 2, strain QB935. The positions of β -glucanase I–IV are shown on the right. For comparison, molecular masses of some comigrated chloroplast thylakoid membrane proteins of *Vicia faba* (Süss & Schmidt, 1982) are indicated in kDa.

Table 2. Characterization of some mutations affecting β -glucanase production of *B. subtilis* 168

Mutants were obtained by NTG treatment of *B. subtilis* 168 *trpC2* as described in the text. All *bgl* mutations tested were transformed in *B. subtilis* QB935 (kit6) by congression with *lys-I* marker. Susceptibility to phage PZA was also tested. In all strains listed in this table, no plaques appeared on plates seeded with about 10^8 p.f.u., as determined in the *spo0A* indicator strain. The enzyme activities are means of three independent experiments, and are expressed as optical units $\text{ml}^{-1} \text{h}^{-1}$.

Strain	<i>bgl</i> mutation	β -Glucanase	α -Amylase	Protease	Cells treated at 80 °C/ untreated cells (numbers $\times 10^{-7}$)	Percentage sporulation
QB935	<i>bgl</i>	34.0	3.00	23.58	203/270	75.2
BG311	<i>bgl-1</i>	3.2	2.95	21.58	193/289	66.8
BG312	<i>bgl-12</i>	3.0	2.25	23.80	166/208	79.8
BG313	<i>bgl-26</i>	3.3	0.48	10.1	20/122	16.4
BG314	<i>bgl-35</i>	3.2	2.25	23.3	228/261	87.4
BG315	<i>bgl-101</i>	2.8	3.00	27.4	150/198	75.8
BG310	<i>bgl-102</i>	2.3	2.70	11.5	34/157	21.7

Bgl^- phenotype is not due to a *spo0* mutation. Moreover, no differences in frequency of sporulation were observed in mutants *bgl-1*, *bgl-12*, *bgl-35* and *bgl-101* compared to the wild-type (Table 2).

Transduction mapping of *bgl* mutations

The position of *bgl* mutations *bgl-1*, *bgl-12*, *bgl-35* and *bgl-101* was mapped by phage PBS1-mediated transduction. Lysates of PBS1 prepared from *bgl-35* were used to transduce separately the nine reference recipients of Dedonder *et al.* (1977). Various markers were selected for, and recombinants were picked up and determined for linkage to *bgl-35* as described in Methods. A weak but significant linkage was shown between *bgl-35* and *ctrA* (15/351). A definite contrasfer was detected between the *bgl-35* mutation and *sacA321* (167/500) and also between *bgl-35* and

Fig. 2. Map location of *bgl* mutations 35, 1, 12 and 101 on the *B. subtilis* chromosome. The map distances shown are expressed as percentage recombination values: percentage recombination = $(1 - \text{cotransfer}) \times 100$. The arrowheads point to the selected markers.

purA16 (121/329). According to Henner & Hoch (1980) *sacA* and *purA* are located at positions 335 and 355, respectively, near to the origin of the *B. subtilis* chromosome. On the basis of cotransfer rates we deduced the position of *bgl-35* to be between these markers in the vicinity of *hutH1* (Henner & Hoch, 1980). A phage PBS1-mediated cross involving strains BG304 (*bgl-35*) and IA272 (*hutH1*) showed that these two markers were closely linked: 73.6% cotransfer was observed. But attempts to demonstrate linkage between the *bgl* and *hutH* marker by DNA-mediated transformation were unsuccessful.

Three other *bgl* mutations, *bgl-1*, *bgl-12* and *bgl-101*, which exhibit the same phenotype as *bgl-35* (see above), were also mapped at about the position of *bgl-35*. Crosses between various *bgl* mutations with *sacA*, *hutH* and *purA* also gave similar linkage data. Thus, all the *bgl* mutations mapped are located within the region between *sacA* and *purA* on the *B. subtilis* chromosome (Fig. 2).

Cloning and expression of a bgl gene from B. subtilis 168

A 3.8 kb DNA fragment containing the gene coding for the 22 kDa β -glucanase of *B. subtilis* 168 was cloned using λ -phage Charon 4A and plasmid pBR322 as vectors and the same strategy as described for cloning of the β -glucanase gene of *B. amyloliquefaciens* (Borriss *et al.*, 1985). A preliminary restriction map (Fig. 3) from the cloned DNA showed close homologies between the genes from *B. subtilis* 168 and *B. subtilis* C120 (Cantwell & McConnell, 1983).

For the construction of a shuttle plasmid carrying the β -glucanase gene and able to replicate also in *B. subtilis*, vector plasmid pHV33 (Primrose & Ehrlich, 1981) was used. The 3.8 kb DNA fragment was ligated into the *EcoRI* site of pHV33. Clones containing Bgl⁺-pHV33 plasmid were selected after transformation of *E. coli* HB101. From transformants exhibiting the Cm^r, Ap^r, Tc^r and Bgl⁺ phenotype, plasmid pHV33-012 was isolated and transformed into *B. subtilis* BG319 carrying chromosomal mutation *bgl-35*. Cm^r transformants, obtained in this experiment, also recovered to produce β -glucanase, suggesting that the *bgl-35* mutation could be complemented by pHV33-012 *in trans*.

Southern blotting confirmed that the 3.8 kb DNA fragment cloned in pHV33 is derived from the chromosomal DNA of *B. subtilis* 168 (results not shown).

In order to avoid interferences caused by integration of homologous DNA into the chromosome we used *B. subtilis* RM125/2 (*recE4*) transformed by pHV33-012 to determine quantitatively the effect of gene dosage on extracellular β -glucanase production in *B. subtilis*. After growth in nutrient broth the productivity of the wild-type strain and a strain harbouring recombinant plasmid pHV33-012 were compared. The results indicated that the ability to produce β -glucanase was greatly enhanced by the presence of recombinant pHV33-012 and was about 20 times higher than in strains without the plasmid under our test conditions (Table 3).

Fig. 3. Map of integration vector pHV60-28. The inserted 3.8 kb fragment is indicated by the hatched double-lined region in the circle. The deleted part of pC194 (double-lined area) codes for Cm^r which is expressed in *E. coli* and *B. subtilis*. The pBR322 moiety which contains the origin of replication and regions responsible for expressing Tc^r and Ap^r in *E. coli* is represented by a single line.

Fig. 4. Proteins from culture fluids of *B. subtilis* RM125/2 with and without pHV33-012. The proteins were separated by electrophoresis in 8–18% polyacrylamide gradient gels (a) then blotted onto nitrocellulose strips prepared with antibodies raised against purified β -glucanase (b) or used for detection of β -glucanase in the gel by sandwiching on a second polyacrylamide gel containing lichenan (c). Lane 1, without plasmid; lane 2, with pHV33-012.

Table 3. β -Glucanase activities of *B. subtilis* strains with and without recombinant plasmid pHV33-012

Cells were grown with shaking for 24 h in nutrient broth at 37 °C. The β -glucanase activity present in the culture fluid was determined as described previously (Borriess, 1981).

Strain	β -Glucanase (U ml ⁻¹)
<i>B. subtilis</i> 168 <i>trpC2</i> (donor strain for 3.8 kb DNA fragment)	0.6
<i>B. subtilis</i> RM125/2 <i>recE4</i> (host strain for pHV33-012)	1.1
<i>B. subtilis</i> RM125/2 <i>recE4</i> (with pHV33-012)	15.8

The increased β -glucanase activity in *B. subtilis* harbouring pHV33-012 was caused by increased synthesis of a 22 kDa protein, which cross-reacted with rabbit antiserum raised against purified *B. subtilis* β -glucanase and had hydrolytic activity on lichenan (Fig. 4).

Fig. 5. Proposed pathway for homologous recombination between integrative plasmid pHV60-28 and the chromosomal region homologous to the *EcoRI* 3.8 kb DNA fragment. The inset shows the results of Southern hybridization using plasmid pHV60-28 as a probe to restriction digests of pHV60-28 and chromosomal DNAs of the parent strain (168) and the strain transformed by pHV60-28 (BG320). Each DNA was digested with *EcoRI* or *AvaI*, electrophoresed in 0.8% agarose, and blotted onto a nitrocellulose filter. Slots: 1, *EcoRI* digest of pHV60-28; 2, *EcoRI* digest of strain BG320; 3, *EcoRI* digest of strain 168; 4, *AvaI* digest of pHV60-28; 5, *AvaI* digest of strain BG320; 6, *AvaI* digest of strain 168. Note that autoradiographs of lanes 1 and 4 were obtained after a shorter exposure time than lanes 2, 3, 5 and 6. The diagram shows the postulated insertion of pHV60-28 by a Campbell model of homologous recombination. The hatched region in the circle represents the cloned 3.8 kb fragment in plasmid pHV60-28; the thick black line is the *bgl*-35 mutant allele. Note that the position of this mutation within the *bgl* coding region has yet not been determined. The bottom double line is the predicted structure of the *B. subtilis* chromosome in the region of the pHV60-28 insertion. Integration and thus *bgl* gene duplication in the chromosome is assumed to occur by a single cross-over between homologous plasmid and chromosomal DNAs. Consequently the plasmid moiety harbouring *Cm^r* becomes flanked by the duplicated *bgl* regions. In one of the duplicated structures the *bgl* gene is restored by replacement of mutation *bgl*-35 by inserted wild-type DNA. Endonuclease restriction sites for *EcoRI* (E), *AvaI* (A) and *PvuII* (P) are indicated. The distances between *EcoRI* sites and between *AvaI* sites are given in kb and correspond to the fragments visualized in the Southern hybridization experiment.

Insertion of a cloned β-glucanase gene into the chromosome

To show unambiguously that the *bgl* mutations mapped on the *B. subtilis* genome between *sacA* and *purA* are mutations within the structural gene encoding the 22 kDa β-glucanase, we applied the plasmid integration technique (Haldenwang *et al.*, 1980) using a recombinant plasmid that expresses its drug resistance in *B. subtilis* but is unable to replicate autonomously in this host. After transformation of this plasmid in *B. subtilis*, drug resistant transformants were selected in which the plasmid was integrated into the chromosome by homologous recombination via cloned *B. subtilis* DNA inserted into the plasmid vector.

To construct an integrative plasmid carrying the cloned 3.8 kb *B. subtilis* DNA fragment we used plasmid pHV60, which only replicates in *E. coli* but expresses chloramphenicol resistance in *E. coli* and in *B. subtilis* (Michel *et al.*, 1983). The 3.8 kb DNA fragment was ligated into the *EcoRI* site of pHV60 and used to transform *E. coli* HB101. Cm^r colonies expressing also the β-glucanase gene were identified by their haloes on lichenan agar. From those colonies plasmid pHV60 was isolated and was shown to contain the 3.8 kb DNA insert. The orientation of the inserted fragment was determined by digestion with restriction enzymes *PvuI* or *AvaI* (Fig. 3). Upon transformation of pHV60-28 into *B. subtilis* BG319 (*bgl-35*), Cm^r and Bgl⁺ clones were obtained, suggesting that the integrated plasmid complements the *bgl-35* mutation. To verify that the plasmid had been inserted into the *B. subtilis* chromosome, Southern hybridization was performed using chromosomal DNAs isolated from parent strain 168 and from the transformants, with nick-translated plasmid pHV60-28 as a probe. As expected, chromosomal DNAs of strain 168 and of the transformants contained the 3.8 kb *EcoRI* fragment, which hybridized to the pHV60-28 probe, indicating the presence of the *bgl* gene. An additional band corresponding to plasmid pHV60 was found in the *EcoRI*-digested chromosomal DNA from Cm^r transformants, indicating the insertion into the chromosome of the integrative plasmid pHV60-28. Using restriction enzyme *AvaI*, plasmid pHV60-28 gave two fragments of about 4.0 and 6.4 kb. Fragments of these sizes were found in the DNA of pHV60-28 transformants, whilst two additional bands were found in the cellular DNA of transformant and wild-type strains, indicating the distances between the internal *AvaI* site to the next *AvaI* sites 'outside' the cloned 3.8 kb DNA fragment on the chromosome to be 2.8 kb and 1.5 kb, respectively (Fig. 5).

The pattern of hybridizing *AvaI*-digested DNA bands from transformants and from the parent strain shows the integration of pHV60-28 into the chromosome to be within the *bgl* gene region.

Mapping of pHV60-28-mediated Cm^r marker on the chromosome

The phage PBS1 transducing lysate was prepared on BG320(pHV60-28) and used to infect strains BG317 (*purA16 bgl-35*) and BG318 (*sacA321 bgl-102*). Cm^r transductants were selected. Cotransduction rates with chromosomal markers were 32/122 (*sacA321/Cm^r*) and 30/100 (*purA16/Cm^r*). The Bgl⁺ phenotype was restored in all of the Cm^r transductants.

The linkage between Cm^r and *bgl* was further proved by DNA-mediated transformation of strain BG319 (Res⁻ Mod⁻ *bgl-35*) using chromosomal DNA prepared from BG320(pHV60-28). Of 138 Cm^r transformants 137 were Bgl⁺, suggesting that the Cm^r marker was cointegrated into the *B. subtilis* chromosome together with the β-glucanase gene.

DISCUSSION

By using a new sensitive method for detection of renatured glucanolytic enzymes on polyacrylamide gradient gels, the presence of four distinct lichenan-hydrolysing enzymes in the culture fluid of *B. subtilis* 168 was established.

Mutants affected in glucanase activity were isolated from mutagenized germinating spores of *B. subtilis*. The only difference observed between the wild-type and one group of mutant strains was the absence of active 22 kDa β-glucanase polypeptide while the glucanolytic activities of three minor enzymes with molecular masses ranging from 33 to 36 kDa were still preserved. Even though a polypeptide in the range of 22 kDa was also present in the culture fluid of mutant strains, no polypeptide cross-reacting material could be detected in these mutant strains.

The minor β -glucanase represented about 5 to 10% of the total lichenan hydrolysis activity in *B. subtilis*, as shown by comparison of the β -glucanase activity of the wild-type with that of mutant strains lacking active 22 kDa β -glucanase. No cross-reactivity could be found between minor β -glucanases and the 22 kDa enzyme, suggesting that the minor glucanases are encoded by gene(s) different from the gene responsible for 22 kDa β -glucanase. However, at present we have no information concerning the enzymic nature of the newly detected minor β -glucanases. Determination of the substrate specificity of the purified enzymes will elucidate whether they are in fact lichenases of type EC 3.2.1.73, as is the 22 kDa β -glucanase.

The fact that all the *bgl* mutations studied could be transferred by single transduction or transformation events into the *bgl*⁺ background makes it highly unlikely that any of these mutants is changed in more than one gene locus. In addition, other properties tested were not changed in mutants *bgl*-1, *bgl*-12, *bgl*-35 and *bgl*-101. Moreover, transfer of a cloned *B. subtilis* DNA fragment coding for β -glucanase in *E. coli* fully restored β -glucanase activity in *B. subtilis* *bgl* mutants. When the β -glucanase gene was inserted into the *Eco*RI site of integrative plasmid pHV60, transformants were phenotypically Bgl⁺ and Cm^r, suggesting that the plasmid was integrated into the chromosome of the *bgl* mutant strain. This was confirmed by Southern hybridization between digested chromosomal DNAs and plasmid pHV60-28 as a probe. From these results we conclude that mutations from the type *bgl*-35 are complemented in *cis* and *trans* by a cloned 3.8 kb DNA fragment, which was shown to encode the 22 kDa β -glucanase I polypeptide.

The position of the *bgl* locus coding for the 22 kDa β -glucanase activity was mapped by transduction analysis. All *bgl* mutations (*bgl*-1, *bgl*-12, *bgl*-35 and *bgl*-101) mapped between *sacA* and *purA*, at about position 345 on the circular chromosomal map of Henner & Hoch (1980), presumably close to *hutH1*. However, while 73.6% cotransfer of *hutH1* and *bgl* mutations was observed in phage PBS1-mediated transduction crosses, no linkage was found between *hutH1* and *bgl* in transformation experiments. The gene order *ctrA-sacA-bgl*(-1, -12, -35, -101)-*purA*, deduced from several multifactor crosses between *bgl* and *ctrA*, *sacA* and *purA* mutations, was also confirmed by the results of mapping Cm^r in strains transformed by the integrative plasmid pHV60-28. Southern hybridization verified that Cm^r of the plasmid was incorporated into the *B. subtilis* chromosome in physical association with the *bgl* coding region by a Campbell-like mechanism (Fig. 5). Rates of cotransfer for Cm^r obtained with *purA* and *sacA* were the same as estimated for *bgl* mutations.

Thus the *bgl* locus responsible for synthesis of 22 kDa β -glucanase maps within a relatively poorly marked area of the *B. subtilis* chromosome and provides a useful tool for genetic mapping in this bacterium. Furthermore, the location of the *bgl* locus also suggests that genes coding for several extracellular enzymes are distributed over the entire chromosome of *B. subtilis* (Henner & Hoch, 1980).

For fine mapping of *bgl* mutations within the *bgl* coding region we intend to do a complementation analysis using deleted derivatives of the integrative plasmid pHV60-28.

We thank Dr R. Dedonder, Dr V. Fučík, the Bacillus Genetic Stock Center and Dr S. D. Ehrlich for providing strains and plasmids. Experiments with phage PZA were done by Dr V. Fučík.

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