Cloning and Expression of a Bacillus subtilis Endo-1,3-1,4-β-D-Glucanase Gene in Escherichia coli K12

By EDWARD HINCHLIFFE

Research Department, Bass PLC, 137 High Street, Burton on Trent DE14 1JZ, UK

(Received 22 September 1983)

EcoRI fragments of DNA from Bacillus subtilis NCIB 8565, a high producer of an endo-1,3-1,4-β-D-glucanase, were 'shot-gun' cloned in the plasmid vector pBR325. A 3.5 kb insert, carrying single restriction sites for AVal, BglII, ClaI, PvuI and PvuII, was shown to direct the synthesis of β-glucanase in Escherichia coli K12. Enzyme activity was demonstrated in extracellular fractions of E. coli harbouring the β-glucanase gene; however, the largest proportion (>50%) of total enzyme activity was periplasmic in location. β-Glucanase activity and cellular location were independent of the orientation of the 3.5 kb fragment in pBR325.

INTRODUCTION

The Gram-positive bacterium Bacillus subtilis produces an extracellular endolytic enzyme (1,3-1,4-β-D-glucan 4-glucanohydrolase; EC 3.2.1.73) capable of hydrolysing alternating sequences of β1,3- and β1,4-linked β-D-glucans (Moscatelli et al., 1961; Rickes et al., 1962). The unique action of this enzyme precludes its ability to hydrolyse repeating sequences of β1,3- or β1,4-linked glucans as in laminarin and carboxymethylcellulose, respectively. In this respect the B. subtilis enzyme behaves like that isolated from malted barley (Barras et al., 1969; Manners & Wilson, 1976). There is considerable interest in the brewing industry in enzymes produced by bacteria and fungi, and their ability to complement the enzymes present in malt (Godfrey, 1983a). Strains of B. subtilis have already been developed for the production of β-glucanase for industrial purposes (Godfrey, 1983b). However, commercial enzyme preparations are impure and often have variable and unpredictable properties (C. W. Bamforth, personal communication). Consequently the cloning, expression and concomitant amplification of a β-glucanase gene will assist in the further characterization and purification of the enzyme for industrial use. In addition, the availability of a cloned gene affords the possibility of modifying brewing yeasts to degrade β-glucan during beer fermentations, thus alleviating problems associated with high levels of β-glucan in beer, such as filtration and the formation of gels and hazes (Bamforth, 1982).

Here I describe the screening of several B. subtilis strains for their ability to hydrolyse barley β-glucan, and report the cloning and expression of a β-glucanase gene from strain NCIB 8565 in Escherichia coli. A B. subtilis gene coding for a β-glucanase has recently been cloned and expressed in E. coli using bacteriophage λ and plasmid vectors; the active gene has been located within a 1·6 kb EcoRI–PvuI DNA fragment and directs the synthesis of an extracellular enzyme in E. coli (Cantwell & McConnell, 1983).

METHODS

Bacterial strains and plasmids. The strains of B. subtilis used were: NCIB 3610, 8057, 8054, 8565, 8646, 10144 (National Collection of Industrial Bacteria); BGSC 2A1, 3A1, 3A11 (Bacillus Genetic Stock Center, Ohio State University, USA); 168 trpC2 (supplied by J. Errington, Dept of Biochemistry, University of Oxford, UK). The abbreviations: Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline.
host strain of E. coli K12 was HB101 (hsdS leu pro lac gal strA thi recA) (Boyer & Roulland-Dussoix, 1969). The E. coli plasmid vector was pBR325 (Bolivar, 1978).

Media and growth conditions. LB medium (Bertani, 1951) was used for the growth of liquid cultures, and was aerated by shaking at 37°C. LB medium was solidified with 1.5% (w/v) agar to make LA plates. When appropriate, LA medium was supplemented with 0-05% (w/v) barleylan. 

Antibiotics. These were obtained from Sigma and were added to media as freshly prepared solutions at the following final concentrations (µg ml⁻¹): ampicillin (Ap), 25; tetracycline (Tc), 15; chloramphenicol (Cm), 10.

Detection and assay for β-glucanase activity. Bacterial colonies were screened for β-glucanase activity by a modification of the Congo Red screening method of Teather & Wood (1982). Bacterial colonies growing on LA plus β-glucan were replica-plated to selective media and the master plate was washed and stained with Congo Red (0-04%, w/v). The colonies producing β-glucanase were surrounded by a zone of clearing against a red-stained agar. A similar method has recently been described by Cantwell & McConnell (1983).

The β-glucanase activity associated with culture supernatants was assayed by the radial diffusion assay of Martin & Bamforth (1983). β-Glucan substrate was either barley β-glucan (Biocon, Eardiston, UK) or Lichenan (from Usnea barbata; Sigma). β-Glucanase activity was determined by measuring the clearing diameter (mm) and converting to arbitrary units as defined by Martin and Bamforth (1983).

Preparations were also assayed for the ability to liberate reducing sugars from β-glucan (barley β-glucan; laminarin, Sigma; carboxymethylcellulose, BDH). Enzyme preparation (0-05-0-1 ml) was added to 0-5 ml β-glucan (2 mg ml⁻¹); the reaction was made to 1 ml with H₂O and incubated at 50°C for 10 min. Reactions were stopped with the addition of 1 ml dinitrosalicylic acid solution (0-25 g 3,5-dinitrosalicylic acid, 5 ml 2 M-NaOH, 12 ml H₂O; 7-5 g potassium sodium tartrate diluted to 25 ml) and boiling for 10 min. After the addition of 5 ml H₂O the amount of reducing sugar was estimated by reading the absorption at 540 nm. One unit of activity was defined as the amount of enzyme capable of liberating 1 µmol reducing sugar min⁻¹ (using maltose as reference) under the conditions described above.

Extracellular, periplasmic and cellular enzyme fractions. These were prepared and defined as described by Cornelis et al. (1982) with the exception that cells were washed in 0-2 M-Na₂HPO₄, 2H₂O, 0-1 M-citric acid, pH 5-6.

Isolation of DNA. Plasmid DNA was isolated by caesium chloride/ethidium bromide equilibrium centrifugation of cleared cell lysates using the method of Clewell & Helinski (1969), with the modifications of Zahn et al. (1977). Chromosomal DNA was isolated from exponentially growing cells following Triton lysis (Katz et al., 1973), with the modifications of Kaiser & Murray (1979). In some experiments a rapid small-scale plasmid extraction procedure was used (Birnboim & Doly, 1980).

Restriction, ligation and electrophoresis of DNA. Restriction endonucleases were purchased from Bethesda Research Laboratories and Boehringer and were used according to the manufacturers' recommendations. Recombinant plasmids containing B. subtilis genomic DNA were obtained by the in vitro ligation of endonuclease EcoRI-generated fragments in the vector pBR325. DNA (25 µg ml⁻¹) was ligated with T₄ DNA ligase (New England Biolabs, Bishop's Stortford, UK; 5-10 units ml⁻¹) at 15°C, in 0-1 M-Tris/HCl (pH 7-2), 0-01 M-EDTA, 0-1 M-MgCl₂, 0-1 M-dithiothreitol and 0-01 M-ATP. DNA fragments produced by endonuclease digestion were analysed by agarose gel electrophoresis (Helling et al., 1974).

Transformation. This was done as described by Wilson & Murray (1979).

RESULTS

Identification of a β-glucanase gene donor

Preliminary screening of ten B. subtilis strains by the radial diffusion method indicated that there was considerable variation in enzyme activity, with clearing zones varying in diameter from 9 to 20 mm on barley β-glucan (10 and > 548 arbitrary units ml⁻¹, respectively). Four strains, listed in Table 1, were identified as having significantly higher β-glucanase activity than the remaining six. Strain NCIB 8565 produced the highest levels of β-glucanase activity, whether determined on barley β-glucan or lichenan. This strain was therefore adopted as the most suitable donor of a β-glucanase gene. When culture supernatants of E. coli HB101 were assayed for β-glucanase activity, there was no detectable zone of clearing, confirming the supposition that E. coli lacks a functional β-glucanase enzyme.

Construction and isolation of recombinant plasmids carrying the β-glucanase gene of NCIB 8565

B. subtilis DNA was cut with EcoRI and ligated to EcoRI-digested pBR325 DNA as described above. The ligated molecules were then transformed into HB101 and the resulting transformants
Table 1. Radial diffusion assay for \(\beta\)-glucanase activity in \(B.\) subtilis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Barley (\beta)-glucan Diameter (mm)*</th>
<th>Activity†</th>
<th>Lichenan Diameter (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGSC 3A11</td>
<td>14</td>
<td>74</td>
<td>9.5</td>
</tr>
<tr>
<td>NCIB 3610</td>
<td>18</td>
<td>367</td>
<td>12.0</td>
</tr>
<tr>
<td>NCIB 8565</td>
<td>20</td>
<td>&gt;548</td>
<td>13.5</td>
</tr>
<tr>
<td>NCIB 8646</td>
<td>18</td>
<td>367</td>
<td>13.0</td>
</tr>
</tbody>
</table>

* 0.1 ml of 16 h LB cultures assayed as described in Methods.
† Arbitrary units of \(\beta\)-glucanase activity ml\(^{-1}\) (according to Martin & Bamforth, 1983).

![Fig. 1. Physical map of plasmid pEHB3, obtained from restriction endonuclease digestion and agarose gel electrophoresis of plasmid DNA.](image)

Restriction mapping of pEHB3

The recombinant plasmid pEHB3 was singled out for a more detailed analysis of its recombinant DNA. Restriction endonuclease digestion with a variety of different enzymes resulted in a preliminary restriction map (Fig. 1). This shows that the 3-5 kb EcoRI fragment
Table 2. Levels of β-glucanase activity and cellular location in HB101(pEHB3) and HB101(pEHB5)

Cellular fractions were assayed for the ability to liberate reducing sugars from barley β-glucan. β-Glucanase activity is expressed as units per ml of culture grown in 50 ml LB Ap medium for 16 h at 37 °C. The data presented indicate the range of values obtained for two independent experiments labelled A and B. Numbers in parentheses represent the mean β-glucanase activity in each fraction expressed as a percentage of the total activity.

<table>
<thead>
<tr>
<th>Cellular fraction</th>
<th>HB101(pEHB3) A</th>
<th>HB101(pEHB3) B</th>
<th>HB101(pEHB5) A</th>
<th>HB101(pEHB5) B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular</td>
<td>0:23</td>
<td>0:5</td>
<td>0:19</td>
<td>0:23</td>
</tr>
<tr>
<td>Periplasmic</td>
<td>0:62</td>
<td>0:99</td>
<td>0:54</td>
<td>0:45</td>
</tr>
<tr>
<td>Cellular</td>
<td>0:20</td>
<td>0:27</td>
<td>0:13</td>
<td>0:25</td>
</tr>
<tr>
<td>Total</td>
<td>1:05</td>
<td>1:76</td>
<td>0:86</td>
<td>0:93</td>
</tr>
</tbody>
</table>

possesses a single asymmetric PvuII site, which was used subsequently to map the orientation of the EcoRI inserts in plasmids pEHB5–7.

Orientation of the 3·5 kb EcoRI fragment in plasmids pEHB5–7

Plasmids pEHB5–7 were digested with the restriction endonuclease PvuII to determine the orientation of insertion of the EcoRI fragment in the EcoRI site of pBR325. PvuII digestion of pEHB6 and pEHB7 generated fragments of approximately 4·2, 2·6 and 2·7 kb, indicating that each carried the EcoRI fragment in the same orientation as pEHB3 (Fig. 1), whereas pEHB5 gave fragments of approximately 6·0, 2·6 and 1·0 kb upon PvuII digestion, indicating that the EcoRI fragment was in the opposite orientation to that described for pEHB3 (Fig. 1).

Substrate specificity of the β-glucanase enzyme produced by E. coli HB101(pEHB3)

The supernatant from an overnight (16 h) culture of HB101(pEHB3) on LB Ap medium was assayed for the ability to hydrolyse barley β-glucan, laminarin (1,3-β-D-glucan) and carboxymethylcellulose (1,4-β-D-glucan). The production of reducing sugars was determined in order to assess the substrate specificity of the enzyme. There was no apparent hydrolysis of either laminarin or carboxymethylcellulose under the conditions described in Methods. However, barley β-glucan was hydrolysed (a value of 0:21 β-glucanase units ml⁻¹), indicating that the enzyme was specific for mixed-linkage 1,3-1,4-β-D-glucan.

Expression and cellular location of β-glucanase activity in E. coli HB101

β-Glucanase activity was estimated in HB101(pEHB3) and HB101(pEHB5), to determine whether the orientation of insertion of the 3·5 kb EcoRI fragment in pBR325 affected the level of enzyme expression and/or cellular location. Extracellular, periplasmic and cellular fractions were assayed for the ability to liberate reducing sugars from barley β-glucan. From Table 2 it appears that HB101(pEHB3) produces approximately 1·6 times more β-glucanase than HB101(pEHB5), under the same conditions. However, there is little difference in the relative location of the enzyme for each strain, the highest activity being associated with the periplasmic fraction (Table 2).

The production of β-glucanase was followed more closely in a culture of HB101(pEHB3), in order to determine the location of the enzyme during cell growth. Samples were taken from the culture at intervals and treated to separate extracellular, periplasmic and cellular activities. There was a rapid increase in total activity as the culture commenced exponential growth (Fig. 2). This increase was followed closely by an initial increase in cellular activity, indicative of enzyme synthesis. As the culture entered late exponential growth the periplasmic and extracellular activity increased at the expense of the cellular, suggesting movement of the newly synthesized enzyme into the periplasm and subsequently the culture supernatant (Fig. 2).
The evidence presented in this paper is consistent with the cloning of a *B. subtilis* β-glucanase gene and its expression in *E. coli*. The enzyme present in cell-free culture supernatants of strain HB101(pEHB3) was specific for mixed linkage 1,3-1,4-β-D-glucan, and in this respect was identical to the endo-1,3-1,4-β-D-glucanase (EC 3.2.1.73) isolated from *B. subtilis* (Moscatelli et al., 1961). The active β-glucanase gene was located on a 3-5 kb EcoRI fragment common to all the recombinant plasmids isolated in HB101. Estimates of β-glucanase activity in HB101(pEHB3) and HB101(pEHB5) indicated that the former produced more β-glucanase, suggesting that the orientation of the EcoRI fragment in pBR325 may be important. The fact that each plasmid was able to direct the synthesis of the enzyme would imply that expression was not brought about by gene fusion with adjacent vector sequences, since a functional hybrid protein would be unlikely to be generated in both orientations. However, the difference in the level of expression between pEHB3 and pEHB5 may be due to the influence of vector sequences acting upon the β-glucanase gene. Whatever the explanation, it seems likely that the β-glucanase gene is directing its own transcription and translation in *E. coli*. A 4 kb EcoRI DNA fragment recently isolated from *B. subtilis* NCIB 2117 has also been shown to express β-glucanase activity in *E. coli*, independently of its orientation in the plasmid vector pBR325 (Cantwell & McConnell, 1983). The β-glucanase gene described by Cantwell & McConnell (1983) has been located on a 1-6 kb EcoRI-PvuII fragment within the 4 kb EcoRI insert. However, comparison of the restriction site data between the two EcoRI fragments shows differences in the relative location of the single AvaI site. In addition, the 3-5 kb EcoRI insert in pEHB3 has single restriction sites for the enzymes *Cla*I and *Pvu*II which are absent in the 4 kb EcoRI fragment (Cantwell & McConnell, 1983). In contrast, the 4 kb EcoRI fragment has a single site for *Sal*I which is not present in pEHB3 (Fig. 1). This would indicate a degree of intraspecific variation in *B. subtilis*. It will be interesting to make a more detailed comparison of the two genes by DNA sequence analysis.

Amylase genes recently isolated from *Bacillus coagulans* (Cornelis et al., 1982) and *B. subtilis* (Yang et al., 1983) express functional gene products in *E. coli*. In the former case the expressed protein was found to reside mainly in the periplasm (Cornelis et al., 1982). Here I describe a similar phenomenon for the β-glucanase enzyme, which appears to be predominantly periplasmic in location, although appreciable activity can be found in extracellular fractions. The pattern of enzyme synthesis displayed in cultures of HB101(pEHB3) is much as one would
expect for an extracytoplasmic enzyme, whose passage from the periplasmic space is impeded by the selective permeability of the outer membrane. The absence of an outer membrane in Gram-positive bacteria precludes the possibility of a direct comparison of cellular location in B. subtilis. However, the observation that the enzyme is able to translocate across the inner membrane into the periplasm suggests that the protein possesses all the necessary information for its own export in E. coli. According to the signal hypothesis of Blobel & Dobberstein (1975), the relevant information for export is contained within a 'signal sequence' in the N-terminus of the protein. Such signal sequences have been found in most secretory proteins (Kreil, 1981). Whether a similar situation exists for the B. subtilis 8-glucanase enzyme remains to be determined.

I would like to thank the directors of Bass PLC for permission to publish this paper. I would also like to thank Mrs W. G. Box for technical assistance, and C. W. Bamforth, S. W. Molzahn and E. F. Walton for many helpful discussions and comments on the manuscript.

REFERENCES

TEATHER, R. M. & WOOD, P. J. (1982). Use of Congo red-polysaccharide interactions in enumeration and

