Evidence That Galactanase A from *Pseudomonas fluorescens* Subspecies *cellulosa* Is a Retaining Family 53 Glycosyl Hydrolase in Which E161 and E270 Are the Catalytic Residues†

Kerynne L. Braithwaite,‡ Tereza Barna,‡ Tracey D. Spurway,‡ Simon J. Charnock,‡ Gary W. Black,‡,§ Neil Hughes,|| Jeremy H. Lakey,⊥ Richard Virden,⊥ Geoffrey P. Hazlewood,▽ Bernard Henrissat,○ and Harry J. Gilbert*,‡

Departments of Biological and Nutritional Sciences, Chemistry, and Biochemistry and Genetics, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K., Department of Cellular Physiology, The Babraham Institute, Babraham, Cambridge CB2 4AT, U.K., and Centre de Recherches sur les Macromolécules Végétales, Centre National Recherche Scientifique, affiliated with the Joseph Fourier University, Grenoble, BP 53, F-38041 Grenoble Cedex 9, France

Received May 27, 1997; Revised Manuscript Received September 12, 1997⊗

ABSTRACT: A genomic library of *Pseudomonas fluorescens* subsp. *cellulosa* DNA was screened for galactanase-positive recombinants. The nine galactanase positive phage isolated contained the same galactanase gene designated *galA*. The deduced primary structure of the enzyme (galactanase A; GalA) encoded by *galA* had a M_r of 42 130 and exhibited significant sequence identity with a galactanase from *Aspergillus aculeatus*, placing GalA in glycosyl hydrolase family 53. The enzyme displayed properties typical of an endo-β1,4-galactanase and exhibited no activity against the other plant structural polysaccharides evaluated. Analysis of the stereochemical course of 2,4-dinitrophenyl-β-galactobioside (2,4-DNPG_2) hydrolysis by GalA indicated that the galactanase catalyzes the hydrolysis of glycosidic bonds by a double displacement general acid−base mechanism. Hydrophobic cluster analysis (HCA) suggested that family 53 enzymes are related to the GH-A clan of glycosyl hydrolases, which have an $(\alpha/\beta)_8$ barrel structure. HCA also predicted that E161 and E270 were the acid−base and nucleophilic residues, respectively. Mutants of GalA in which E161 and E270 had been replaced with alanine residues were essentially inactive against galactan. Against 2,4-DNPG_2, E161A exhibited a much lower K_m and k_{cat} than native GalA, while E270A was inactive against the substrate. Analysis of the pre-steady-state kinetics of 2,4-DNPG_2 hydrolysis by E161A showed that there was an initial rapid release of 2,4-dinitrophenol (2,4-DNP), which then decayed to a slow steady-state rate of product formation. No pre-steady-state burst of 2,4-DNP release was observed with the wild-type enzyme. These data are consistent with the HCA prediction that E161 and E270 are the acid−base and nucleophilic catalytic residues of GalA, respectively.

Glycosyl hydrolases cleave their target substrates by either a single or double displacement general acid−base mechanism (*1*). In the active site of these enzymes are two carboxylic acids residues that participate in catalysis, a nucleophile and an acid/base catalyst (*2*). Hydrophobic cluster analysis (HCA)[1] has grouped these enzymes into families. Enzymes in the same family have a similar three-dimensional structure reflecting their common ancestral origin (*3*). The key catalytic residues in enzymes from the same family also appear to be completely conserved. Recently, HCA and X-ray crystallographic data have shown that there is a structural and evolutionary relationship between several different glycosyl hydrolase families; thus, related families have now been grouped into clans (*4*). In addition to containing catalytic domains, it is apparent that some glycosyl hydrolases, such as cellulases and xylanases, that attack the plant cell wall are modular enzymes consisting of a discrete catalytic domain and cellulose binding domain (*5*−*9*).

Although substantial progress has been made in the elucidation of the three-dimensional structures and catalytic mechanisms of cellulases and xylanases, there is a critical lack of information on the structure and mode of action of other important plant cell wall hydrolases, such as those that cleave the side chains of pectin. Pectins, which are major matrix polysaccharide components of the plant cell wall, can consist of either a polymer of unsubstituted polygalacturonic acid residues or a backbone of galacturonic acid and rhamnose residues that are attached to galactan and arabinan side chains. The galactans are polymers of β-1,4-linked galatopyranose units consisting of up to 60 residues (*10*). The side chain components of pectins increase the hydration state of the polysaccharide, preventing the formation of crystalline structures by disrupting interchain backbone interactions, and play a critical role in the binding of pectins

† Financial support was obtained from the Biotechnological and Biological Sciences Research Council (LE13/138), the Overseas European Community Development Fund, and the Royal Society.

* Corresponding author.

‡ Department of Biological and Nutritional Sciences, University of Newcastle upon Tyne.

§ Present address: School of Health Sciences, University of Sunderland, Fleming Building, Wharncliffe Street, Sunderland SR2 3SD, U.K.

|| Department of Chemistry, University of Newcastle upon Tyne.

⊥ Department of Biochemistry, University of Newcastle upon Tyne.

▽ The Babraham Institute.

○ Centre de Recherches sur les Macromolécules Végétales.

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1997.

[1] Abbreviations: 2,4-DNP, 2,4-dinitrophenol; 2,4-DNPG_2, 2,4-dinitrophenyl β-galactobioside; GalA, galactanase A; HCA, hydrophobic cluster analysis; LB, Luria broth.

S0006-2960(97)01239-7 CCC: $14.00

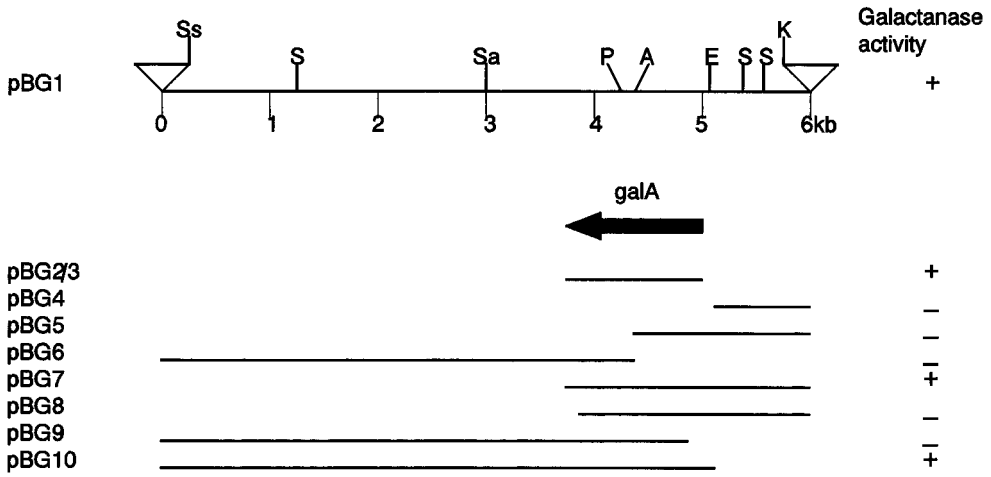

FIGURE 1: Restriction map of recombinant plasmids containing *galA*. The cleavage sites for the restriction enzymes *Apa*I (A), *Eco*RV (E), *Kpn*I (K), *Pst*I (P), *Sma*I, and *Sst*I are shown. The multiple cloning region (MCR) of pBluescriptSK$^-$ in pBG1 is indicated by a ∇, and the restriction sites in the MCR proximal to the pseudomonad insert are shown. The plasmids pBG4−6 were derived by subcloning restriction fragments from the *Pseudomonas* insert in pBG1 into pUC-based vectors. pBG2 and pBG3 were derived by cloning the PCR product encoding mature GalA into pET16a and pET32a, respectively. The plasmids pBG7−10 were truncated derivatives of pBG1 generated by exonuclease treatment. The location and orientation of *galA* in pBG1 is indicated by an arrow. The capacity of the plasmids to express a functional galactanase is indicated (±).

to other polysaccharides within the plant cell wall (*11*). Type-1 galactans are hydrolyzed by endo-β1,4-galactanases (galactanase; EC.3.2.1.89). These enzymes have been isolated from numerous microorganisms including the aerobic fungi *Aspergillus aculeatus* and *Aspergillus niger* and the bacterium *Bacillus subtilis* (*12*, *13*).

Currently, there is a paucity of data on the structure/function relationships of galactanases. The primary structure of only a single galactanase has been described. The enzyme, from *A. aculeatus*, exhibited significant homology with the product of a *Bacillus circulans* open reading frame (ORF) with no known function (*14*). The fungal galactanase, which had a simple molecular architecture that lacked the noncatalytic domains prevalent in a range of plant cell wall hydrolases, was assigned to glycosyl hydrolase family 53. There have been no reports on the amino acid sequence or molecular architecture of a prokaryotic galactanase, and the mechanism by which these enzymes cleave glycosidic bonds has not been elucidated. It also remains to be established whether prokaryotes contain multigene families encoding galactanases, as is the case for cellulases and xylanases (*15*, *16*), or whether the enzyme is the product of a single copy gene. In this paper, we describe the primary structure, biochemical properties, and mechanism of action of the endo-β1,4-galactanase expressed by *Pseudomonas fluorescens* subsp. *cellulosa*.

MATERIALS AND METHODS

Microbial Strains, Nucleic Acids, and Culture Conditions. *P. fluorescens* subsp. *cellulosa* and *Escherichia coli* were cultured in Luria broth (LB) at 37 °C, unless otherwise stated. λ bacteriophage was grown on NZY medium. In galactanase induction experiments, *P. fluorescens* subsp. *cellulosa* was cultured in LB, supplemented with the appropriate sugar or polysaccharide at a final concentration of 0.25% for 12 h at which point the cultures were in mid-exponential phase. Ampicillin (100 μg/mL) and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (80 μg/mL) were added to media to culture *E. coli* transformants and select for pMTL- or λ*ZAPII*-derived recombinants, respectively. To select for galactanase-positive bacteria, LB-agar was supplemented with 0.2% AZCL-galactan (Megazyme Ltd., Ireland). To induce galactanase expression from pET-based plasmids, *E. coli* cultures were grown to mid-exponential phase (A_{550} 0.5) at 30 °C in LB supplemented with ampicillin, isopropyl thio-β-D-galactopyranoside (IPTG) was then added to a final concentration of 500 μM, and induction was allowed to proceed for 4 h. Recombinant λ phage were grown on NZY medium seeded with the *E. coli* strain XL1-Blue. The *E. coli* strains used in this study were JM101, JM83 (*17*), BL21 (*18*), XL1-Blue (Stratagene), and the *dut*$^-$ *ung*$^-$ strain CJ236 (Bio-Rad). The plasmid vectors used in this study were pMTL20−pMTL23 (*19*), pBluescriptSK$^-$ (Stratagene), and pET32a (Novagen). The bacteriophage vectors employed were M13mp19 (*17*) and λ*ZAPII* (Stratagene).

Screening of a Genomic Library for Galactanase-Positive Phage. A genomic library of *P. fluorescens* subsp. *cellulosa* DNA, constructed in λZAPII (*20*) was plated out on NZY top agarose supplemented with the insoluble polysaccharide AZCL-galactan, to a density of 20 plaques/cm^2. After incubation for 16 h at 37 °C, galactanase-expressing phage were surrounded by blue halos in a background containing particles of AZCL-galactan. Galactanase-positive phage were subjected to two rounds of purification after which all the phage screened were galactanase-positive.

Recombinant DNA Technology. DNA-modifying enzymes such as restriction endonucleases and T4 DNA ligase were used as suggested by the manufacturers. Nucleic acid electrophoresis, Southern blotting, plaque hybridization, and transformation of *E. coli* were carried out as described previously (*21*). Plasmid and phage DNA were purified using Qiagen columns. To determine the nucleotide sequence of the galactanase gene, nested deletions of the *Pseudomonas* insert in pBG1 (Figure 1) were generated using the S1/exonuclease kit supplied by Promega. The *Pseudomonas* DNA in the truncated plasmids was sequenced using the PRISM ready reaction dye-deoxy terminator cycle sequencing kit supplied by Applied Biosystems Instruments (ABI) and the ABI 373 automatic sequencing model. Sequences were compiled and ordered using computer

programs described by Staden (*22*). The cloned DNA was sequenced completely in both strands. To select for mutants, DNA was sequenced using the Sequenase version 2.0 kit (Amersham International) employing primers that annealed to appropriate regions of the galactanase gene. To clone the region of the galactanase gene that encoded the mature enzyme into the expression vector pET32a, the Polymerase Chain Reaction (PCR) was employed to amplify the appropriate region of pBG1. The conditions for PCR were as described previously (*23*). The primers used to amplify the DNA were 5′ CGCCCATGGATAATACGCCATTCTATGTG 3′ and 5′ GCGGAATTCATTTATTCGGCTTGTGCGC 3′. The amplified DNA was digested with *Nco*I and *Eco*RI and cloned into the *Nco*I and *Eco*RI sites of pET32a to generate pBG3. Site-directed mutagenesis was carried out using the method of Kunkel (*24*). Mutations were introduced into the galactanase gene and thus into the encoded enzyme by using the following primers to synthesise the DNA *in vitro*: E161A, 5′ TCAATATTGGT**T**GCGTTGCCGACTTG 3′; E270A, 5′ GATAAGCCGTC**G**CCACAATCATGA 3′. The mutated nucleotides are in bold. The target DNA consisted of the amplified galactanase gene, which was cloned initially into *Nco*I/*Eco*RI-restricted pMTL23 and was then excised from the resultant plasmid on a 1.1 kb *Eco*RI/*Hin*dIII restriction fragment and inserted into M13mp19.

Galactanase Purification. Native and mutants of the *Pseudomonas* galactanase were expressed as thioredoxin fusion proteins that contained a $(His)_6$ tag at the N-terminus. The proteins were therefore purified by nickel affinity chromatography using the following method. Cultures (2.4 L) of appropriate recombinant strains of *E. coli* BL21 plysS were grown as described above. The *E. coli* cells were then pelleted, resuspended in 90 mL of 20 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl (buffer A), and disrupted by sonication, and the insoluble material was pelleted by centrifugation at 30000*g* for 30 min. The cell-free extract generated was then applied to a 30 × 10 mm Talon resin column (Novogen) equilibrated with buffer A. Once the bacterial proteins had been applied, the column was washed with 10 vol of buffer A containing 10 mM imidazole. The galactanase fusion protein was then eluted with 30 mL of buffer A containing 15 mM imidazole. The GalA fusion protein eluted from the column was then cleaved with thrombin using the following conditions: three units of thrombin (Novogen) were incubated with three mg of the fusion protein for 16 h in 20 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl and 2.5 mM $CaCl_2$ at 21 °C. Under these conditions, the fusion protein was completely digested into GalA and thioredoxin, respectively. The cleaved protein was then reapplied to the Talon resin column. GalA was recovered in the void volume while thioredoxin, which still contains the $(His)_6$ tag, was bound to the matrix. Recovered GalA was dialyzed against 3 × 1000 vol of 10 mM Tris-HCl buffer, pH 8.0. The purified galactanase was homogenous, as judged by SDS/PAGE.

Synthesis of 2″,4″-Dintrophenyl-β-galactobioside (2,4-DNPG$_2$)

1,2,3,6,2′,3′,4′,6′-Octa-O-acetylgalactobiose. Galactan (2 g) was dissolved in 20 mL of 300 mM HCl and incubated at 100 °C for 2 h. The solution was passed through Dowex-1-AcO^- and evaporated to a syrup. Acetic acid (20 mL) was added, and the mixture was again evaporated to remove traces of water. Acetic anhydride (30 mL) and 5 g of sodium acetate were added to the residue, and the mixture was heated with stirring in a boiling water bath for 1 h. Solvents were evaporated, the residue was shaken with 100 mL of water and 100 mL of CH_2Cl_2, and the mixture was filtered through Hyflo. The layers were separated, and the aqueous fraction was re-extracted with 50 mL of CH_2Cl_2. The combined organic extracts were dried over $MgSO_4$, filtered, and evaporated to give a syrup that was dissolved in 50 mL of toluene and re-evaporated to remove the last traces of acetic acid, to leave a syrup (3.6 g). The syrup was chromatographed on silica. Elution with light petroleum−ethyl acetate (1:1) gave a mixture of acetylated galactooligosaccharides, while elution with light petroleum−ethyl acetate (1:2) gave 0.49 g of 1,2,3,6,2′,3′,4′,6′-octa-*O*-acetylgalactobiose. ^{1}H-NMR spectroscopy suggested that the compound was mainly the β-anomer; ^{1}H-NMR ($CDCl_3$): δ 6.57 (d, 0.27H, $J_{1,2}$ 4.0Hz, H-1α), 5.65 (d, 0.73H, $J_{1,2}$ 8.3 Hz, H-1β). The remaining data are for the β-anomer: δ 5.37 (dd, $J_{3',4'}$ 3.4 Hz, $J_{4',5'}$ 1.2 Hz, H-4′), 5.26 (dd, $J_{1',2'}$ 7.9 Hz, $J_{2',3'}$ 10.6 Hz, H-2′), 5.20 (dd, $J_{2,3}$ 10.2 Hz, H-2), 5.00 (dd, $J_{2',3'}$ 10.3 Hz, H-3′), 4.94 (dd, $J_{3,4}$ 3.1 Hz H-3), 4.38 (dd, $J_{5,6a}$ 4.5 Hz, $J_{6a,6b}$ 12.1 Hz, H-6a), 4.18 (dd, $J_{5,6b}$ 7.1 Hz, H-6b), 4.14 (dd, $J_{4,5}$ 1.0 Hz, H-4), 4.08 (m, 2H, H-6a′ and H-6b′), 3.85 (bt, 2H, H-5 and H-5′), 2.19, 2.15, 2.12, 2.11, 2.07, 2.05, 2.03, 2.00 (8 × s, 8 × Ac).

2″,4″-Dinitrophenyl 2,3,6,2′,3′,4′,6′,-hepta-O-acetyl-β-galactobioside. To remove the acetate group at carbon one, a mixture of hydrazine hydrate (27 μL, 570 μmol) and acetic acid (33 μL, 580 μmol) in *N*,*N*-dimethylformamide (0.5 mL) was added to a solution of the above octaacetate (0.27 g, 400 μmol). After incubating at 20 °C for 40 min, ethyl acetate (10 mL) was then added, and the mixture was washed twice with dilute HCl and then twice with Na_2CO_3 and dried over $MgSO_4$. Filtration and evaporation of the solvent gave 2,3,6,2′,3′,4′,6′-hepta-*O*-acetylgalactobiose as a chromatographically homogenous syrup (0.23 g, 360 μmol, 90%). A mixture of the heptaacetate (0.19 g, 300 μmol), 1,4-diazabicyclo[2,2,2]octane (0.11 g, 1 mol), and 4-Å molecular sieves (1 g) in 2 mL of *N*,*N*-dimethylformamide was stirred at 20 °C for 1 h and, after the addition of 77 mg (50 μL, 410 μmol) of 1-fluoro-2,4-dinitrobenzene, the mixture was incubated for 16 h at the same temperature. Ethyl acetate (20 mL) was then added, and the mixture was filtered to remove the molecular sieves. The filtrate was washed twice with dilute HCl, a dilute solution of Na_2CO_3 and then dried over $MgSO_4$. Filtration and evaporation left a residue (0.2 g) that was chromatographed on silica and eluted with light petroleum−ethyl acetate (2:3) to give first a high running impurity and then the title compound, which was obtained as a gum (0.16 g, 200 μmol, 66%) $[\alpha]_D$ +39° (CH_2Cl_2); ^{1}H-NMR ($CDCl_3$): δ 8.61 (d, $J_{3'',5''}$ 2.7 Hz, H-2″), 8.41 (dd, $J_{5'',6''}$ 9.3 Hz, H-5″), 7.56 (d, H-6″), 5.41 (dd, $J_{1,2}$ 8.1 Hz, $J_{2,3}$ 10.1 Hz, H-2), 5.34 (bd, $J_{3',4'}$ 3.0 Hz, $J_{4',5'}$ ca. 1 Hz, H-4′), 5.21 (dd, $J_{1',2'}$ 7.9 Hz, $J_{2',3'}$ 10.6 Hz, H-2′), 5.12 (d, H-1), 4.97 (dd, H-3′), 4.95 (dd, $J_{3,4}$ 2.7 Hz, H-3), 4.40 (d, H-1′), 4.36 (dd, $J_{5,6a}$ 4.1 Hz, $J_{6a,6b}$ 12.2 Hz, H-6a), 4.28 (dd, $J_{5,6b}$ 7.4 Hz, H-6b), 4.18 (bd, $J_{4,5}$ ca. 1 Hz, H-4), 4.08 (dd, $J_{5',6a'}$ 6.8 Hz, $J_{6a'6b'}$ 11.3 Hz, H-6a′), 4.05 (dd, $J_{5',6b'}$ 6.3 Hz, H-6b′), 3.89 (bdd, H-5), 3.83 (bt, H-5′).

2″,4″-Dinitrophenyl-β-galactobioside. A solution of the above glycoside heptaacetate (80 mg, 100 μmol) in 0.5 mL

```
GAGCCTTACAAAACCTATGGACTGGAGTCCAATAACCCGGCCCATATCAACCGATATGAG

                                                    -100
GGAGTTCGGGACGCGCTACAGTGTAGGTATCAACTGGAAGCTGTAAAACATTTCTGTCCC

GTTGTGTTTTGGCGGCTGCTTCGGCAGCCGCGTTTTTTTGCGGCAATGACTTATAACAAG

                         +1
AACACACCAAAGAGAAGTGAAATGAAAAAAAAGATTCTAGCGGCAACCGCCATTCTGCTG
                        M  K  K  K  I  L  A  A  T  A  I  L  L

                                                              200
GCTGCTATTGCCAATACCGGTGTGGCTGATAATACGCCATTCTATGTGGGCGCCGACCTT
 A  A  I  A  N  T  G  V  A  D  N  T  P  F  Y  V  G  A  D  L

TCCTATGTGAATGAAATGGAAAGCTGCGGAGCCACCTACCGCGACCAGGGTAAAAAAGTA
 S  Y  V  N  E  M  E  S  C  G  A  T  Y  R  D  Q  G  K  K  V

                                                    300
GACCCCTTCCAACTGTTTGCCGATAAAGGCGCCGACCTGGTGCGTGTCCGCCTCTGGCAT
 D  P  F  Q  L  F  A  D  K  G  A  D  L  V  R  V  R  L  W  H

AACGCTACCTGGACAAAATATTCAGACCTGAAAGATGTGAGCAAAACGCTCAAGCGCGCA
 N  A  T  W  T  K  Y  S  D  L  K  D  V  S  K  T  L  K  R  A

                        400
AAAAATGCCGGAATGAAAACCCTGCTGGATTTCCATTATTCCGACACCTGGACCGATCCC
 K  N  A  G  M  K  T  L  L  D  F  H  Y  S  D  T  W  T  D  P

                                                              500
GAAAAACAATTTATTCCCAAGGCCTGGGCCCATATTACCGACACCAAAGAGCTGGCCAAA
 E  K  Q  F  I  P  K  A  W  A  H  I  T  D  T  K  E  L  A  K

GCACTCTACGACTACACCACCGACACCCTGGCCAGCCTCGACCAACAACAGCTGTTACCC
 A  L  Y  D  Y  T  T  D  T  L  A  S  L  D  Q  Q  Q  L  L  P

                                                    600
AACCTGGTACAAGTCGGCAACGAAACCAATATTGAAATCCTGCAGGCGGAAGACACGCTG
 N  L  V  Q  V  G  N  E  T  N  I  E  I  L  Q  A  E  D  T  L

GTACATGGCATTCCCAATTGGCAGCGCAATGCCACACTGCTTAACAGTGGTGTCAACGCT
 V  H  G  I  P  N  W  Q  R  N  A  T  L  L  N  S  G  V  N  A

                        700
GTGCGCGATTACAGTAAAAAAACCGGCAAGCCCATCCAGGTGGTACTGCATATCGCCCAG
 V  R  D  Y  S  K  K  T  G  K  P  I  Q  V  V  L  H  I  A  Q

                                                              800
CCGGAAAATGCCCTCTGGTGGTTTAAACAAGCCAAGGAAAATGGCGTTATTGACTATGAT
 P  E  N  A  L  W  W  F  K  Q  A  K  E  N  G  V  I  D  Y  D

GTAATCGGCCTTTCCTACTATCCGCAGTGGTCTGAATACAGCTTGCCACAATTGCCCGAT
 V  I  G  L  S  Y  Y  P  Q  W  S  E  Y  S  L  P  Q  L  P  D

                                                    900
GCCATTGCCGAATTACAAAATACCTATCACAAACCGGTCATGATTGTGGAGACGGCTTAT
 A  I  A  E  L  Q  N  T  Y  H  K  P  V  M  I  V  E  T  A  Y

CCCTGGACACTGCACAACTTCGACCAGGCCGGTAATGTGTTGGGTGAAAAGGCGGTACAA
 P  W  T  L  H  N  F  D  Q  A  G  N  V  L  G  E  K  A  V  Q

                       1000
CCCGAATTTCCCGCCAGCCCGCGCGGACAATTAACCTATTTGCTCACCCTGACACAATTG
 P  E  F  P  A  S  P  R  G  Q  L  T  Y  L  L  T  L  T  Q  L
```

FIGURE 2: Nucleotide sequence of *galA* and the deduced primary structure of GalA. The nucleotide sequence of *galA* appears in the EMBL database under the accession number X91885.

of CH_2Cl_2 was added to a solution of HCl (from 0.04 mL of acetyl chloride) in 1 mL of MeOH and left for 48 h at 20 °C. Ether (1 mL) was added dropwise to the solution when crystallization of the product occurred. This was filtered off and washed with MeOH−CH_2Cl_2−ether (1:1:1) to give the galactobioside (30 mg, 60 μmol, 60%), mp 164 °C (dec), $[\alpha]_D$ −56° (H_2O), ^{1}H-NMR (D_2O) (partial): δ 8.88 (d, $J_{3'',5''}$ 2.7 Hz, H-3″), 8.52 (dd, $J_{5'',6''}$ 9.3 Hz, H-5″), 7.66 (d, H-6″), 5.40 (d, *J* 8.0 Hz, H-1), 4.62 (d, *J* 7.9 Hz, H-1′); ^{13}C-NMR (D_2O): δ 155.5,142.6, 140.1, 131.0, 123.4, and 118.8 (ArC), 105.4 and 101.7 (C-1,1′), 77.8, 76.3 (2C), 74.0 (2C), 72.6, 71.1, and 69.8 (C-2,2′,3,3′,4,4′,5,5′), 61.8 and 61.5 (C-6,6′).

Enzyme and Protein Assays. The sources of the substrates used in this study were as follows: AZCL-galactan, Azo-galactan, lupin galactan, and potato galactan were purchased from Megazyme Ltd. Except for 2,4-$DNPG_2$, which was prepared as described above, all arylglycosides were obtained from Sigma Chemical Co. Unless otherwise stated, enzyme assays were carried out in 50 mM sodium phosphate buffer, pH 7.0. When measuring the hydrolysis of 2,4-$DNPG_2$, the accumulation of the product, 2,4-dinitrophenol (2,4-DNP), was monitored at 400 nm using an extinction coefficient of 10356 M^{-1} cm^{-1}. The hydrolysis of the other arylglycosides was measured as described previously (*20*). Galactanase activity using lupin or potato galactan was monitored by measuring the release of reducing sugar (*25*). The initial velocity of enzyme reactions was determined over not more than the first 10% of the total reaction. Enzymic hydrolysis of Azo-galactan was monitored by measuring the release of ethanol-soluble dyed galactooligosaccharides. In this assay the galactanase was incubated with 0.5% of the dyed substrate in a reaction volume of 500 μL for 10 min, after which time 2 vol of 99% ethanol (v/v) was added, the precipitated polysaccharide was removed by centrifugation, and the A_{590} of the supernatant was measured. Pre-steady-state kinetics, using 2,4-$DNPG_2$ as the substrate, were performed using a stopped-flow apparatus (Applied Photophysics Model SX-17MV) essentially as described by Bolam et al. (*23*). To identify the products released from galactan by the galactanase, the following reaction was carried out: a 0.2% solution of potato galactan in 50 mM sodium phosphate buffer, pH 7.0, was incubated at 37 °C with 2 units of purified galactanase in a total volume of 2 mL for up to 240 min. At regular time intervals aliquots (20 μL) were removed and boiled for 5 min, and the oligosaccharides present were determined by HPLC as described previously (*26*) except that the sodium acetate gradient was 0−50 mM. Protein concentration (*27*) and xylanase activity (*8*) were measured as described previously.

Zymogram Analysis. Samples for zymogram analysis were subjected to SDS/PAGE essentially as described by ref 28. Fractionated proteins were renatured by incubating the SDS/PAGE gel for 30 min in a 1% (w/v) aqueous solution of Triton X-100 and washing once with distilled water and a further five times for 15 min, each wash with 10 mM sodium phosphate buffer, pH 6.6. The gel was then laid onto an agar replica containing 10 mg/mL potato galactan and 15 mg/mL agar in distilled water, covered with cling film to prevent drying out, and incubated at 37 °C for 16 h. The agar replica was then stained with Congo Red (1%, w/v) and destained with 1 M NaCl. The SDS/PAGE gel was stained with Coomassie Blue.

Stereochemical Course of 2,4-$DNPG_2$ Hydrolysis. The ^{1}H-NMR spectra were recorded at 200 MHz using a Bruker Spectrospin WM 300 spectrometer. Chemical shifts are given in parts per million (ppm) relative to acetone (2.20 ppm). All samples were then redissolved in 0.5 mL of D_2O to a final concentration of 35 mM (17 μmol in total) in a 5-mm NMR tube. After the accumulation of the initial spectra of the three sugars (galactose, galactobiose, and 2,4-$DNPG_2$), the galactanase (10 units), which had been lyophilized and redissolved in D_2O, was added to the NMR tube containing 2,4-$DNPG_2$, which was then placed in the spectrometer probe; spectra were recorded at regular time intervals.

Biophysical Measurements. Circular dichroism (CD) spectra of native and mutant forms of the galactanase were recorded at 20 °C using a Jobin-Yvon CD6 spectrometer using a 0.2-mm path length cuvette. Mean $\Delta\epsilon$ values were calculated using the predicted protein sequences and concentrations derived from absorption measurements at 280 nm on a Cary 4E absorption spectrometer.

HCA. HCA plots were produced, manipulated, and analyzed as described by Henrissat et al. (*4*).

RESULTS

P. fluorescens Hydrolyzes β1,4-Galactan. Previous studies have shown that *P. fluorescens* subsp. *cellulosa* is capable

of degrading a range of plant structural polysaccharides (*15*, *16*, *20*, *29*). To evaluate whether the bacterium has the capacity to hydrolyze β1,4-galactan, the pseudomonad was grown on solid medium containing AZCL-galactan. After 16 h at 37 °C small colonies, surrounded by blue halos, were clearly visible, indicating that the bacterium was capable of degrading β1,4-galactan. To investigate whether the galactanase-degrading enzyme(s) were constitutive or inducible, *P. fluorescens* subsp. *cellulosa* was grown in liquid medium containing different carbon sources. Galactanase activity was induced by either galactose or galactan, but not by the other major plant structural polysaccharides. Glucose repressed the expression of galactanase activity (data not shown). When the pseudomonad was cultured in medium containing galactan, in excess of 90% of total galactanase activity was in the culture supernatant (data not shown), indicating that galactan-degrading enzymes were primarily extracellular.

P. fluorescens subsp. cellulosa Expresses a Single endo-β1,4-Galactanase. To investigate the number of *P. fluorescens* subsp. *cellulosa* genes that encode galactanase activity, a genomic library of the *Pseudomonas* DNA, constructed in *λZAPII*, was screened for galactanase-expressing recombinants. From the 40 000 phage analyzed, 12 displayed significant galactanase activity. The *Pseudomonas* insert from one of the recombinant phage was excised into pBluescript SK$^-$ to generate pBG1. *E. coli* cells containing pBG1 expressed a functional galactanase defined as galactanase A (GalA). The restriction map of the *Pseudomonas* insert in pBG1 is displayed in Figure 1. To locate the approximate position of the galactanase gene in pBG1, various regions of the plasmid were subcloned into other vectors, and the retention of the galactanase-positive phenotype was evaluated. The data showed that the GalA gene, designated *galA*, was located between the *Sal*I and *Eco*RV restriction sites at positions 3.0 and 5.1, respectively.

To investigate whether *galA* was a single copy gene, *P. fluorescens* subsp. *cellulosa* genomic DNA was subjected to Southern hybridization, using *galA* as the probe. The probe hybridized to a single 17 kb *Sal*I restriction fragment and two *Eco*RV (6.0 and 1.5 kb) and *Sal*I restriction fragments (6.0 and 3.5 kb), indicating that *galA* was a single copy gene. Plaque hybridization experiments indicated that the other 11 galactanase-positive phage isolated from the *Pseudomonas* genomic library also contained *galA*. These data indicate that *P. fluorescens* subsp. *cellulosa* contains a single galactanase gene.

Primary Structure of GalA. To determine the nucleotide sequence of *galA* and the primary structure of GalA, the *Pseudomonas* insert in pBG1 was sequenced in both strands. The data (Figure 2) revealed a single open reading frame (ORF) of 1131 bp encoding a protein of M_r 42 130. The ORF had a G + C content of 51.4% and a codon utilization that was similar to other *P. fluorescens* subsp. *cellulosa* genes encoding plant cell wall hydrolases. Several lines of evidence indicated that the ORF constituted *galA*; 7 bp upstream of the putative initiation codon is the sequence AGAGA, which is similar to prokaryotic ribosome binding sequences. The deduced N-terminal sequence of GalA displayed many of the features typical of a bacterial signal peptide; the N-terminal four residues, which consisted of a basic hydrophilic region, were followed by an 18 amino acid sequence that consisted primarily of small hydrophobic residues. The predicted cleavage site of the signal peptide, according to the rules of Perlman and Halvorson (*30*), is between A22 and D23. The deduced M_r of mature GalA (40 500) is similar to the size of the recombinant galactanase expressed by *E. coli* cells harboring pBG1 (Figure 3). The primary structure of GalA did not reveal hydroxy amino acid- or glycine-rich linker sequences typical of modular plant cell wall hydrolases. In addition, GalA did not contain sequences that exhibited identity with the cellulose binding domains present in *Pseudomonas* cellulases and xylanases, which is consistent with the observation that the galactanase did not bind to insoluble forms of cellulose or xylan (data not shown). Truncated derivatives of GalA lacking either 35 or 45 residues from the N- and C-terminus, respectively, were catalytically inactive. Collectively, these data indicate that the galactanase is a non-modular protein consisting of a single catalytic domain.

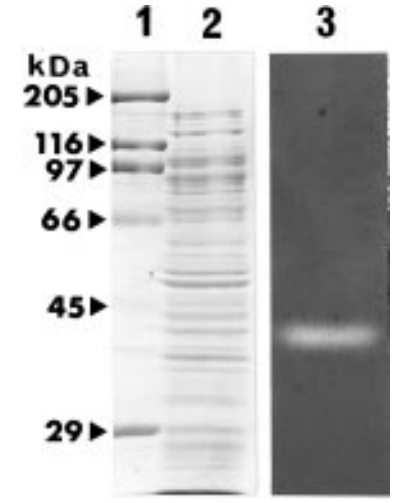

FIGURE 3: Zymogram analysis of GalA. Cell free extract from *E. coli* harboring pBG1 was subjected to zymogram analysis as described in Materials and Methods. Protein size markers (kDa) and cell-free extract proteins are shown in lanes 1 and 2, respectively, after Coomassie Blue staining. Galactanase activity contained in the cell-free extract is depicted in the Congo Red stained agar replica (lane 3).

Comparison of the deduced primary structure of GalA with sequences in the SWISSPROT database showed that the protein exhibited 30% and 35% homology, respectively, with the *Aspergillus aculeatus* endo-β1,4-galactanase and an ORF from *Bacillus circulans*, of unknown function (Figure 4). Using the classification of Henrissat and Bairoch (*31*), GalA appears to belong to glycosyl hydrolase family 53.

Hyperexpression and Characterization of GalA. The level of GalA expression in *E. coli* cells containing pBG1 was very low. To increase the synthesis of the enzyme in the enteric bacterium, *galA* was initially cloned into the expression vector pET16b to generate pBG2. Although *E. coli* cells harboring pBG2 expressed high levels of the galactanase, the enzyme formed inclusion bodies from which the active enzyme could not easily be recovered (data not shown). In an alternative strategy, *galA* was cloned into pET32a to generate the plasmid pBG3 that contains a hybrid gene encoding a thioredoxin-GalA fusion protein. The rationale for this approach is that thioredoxin, when linked to other polypeptides, appears to prevent the formation of inclusion bodies by a mechanism that remains to be elucidated. *E. coli* cells harboring pBG3 expressed significant quantities of soluble and catalytically activity thioredoxin-GalA fusion

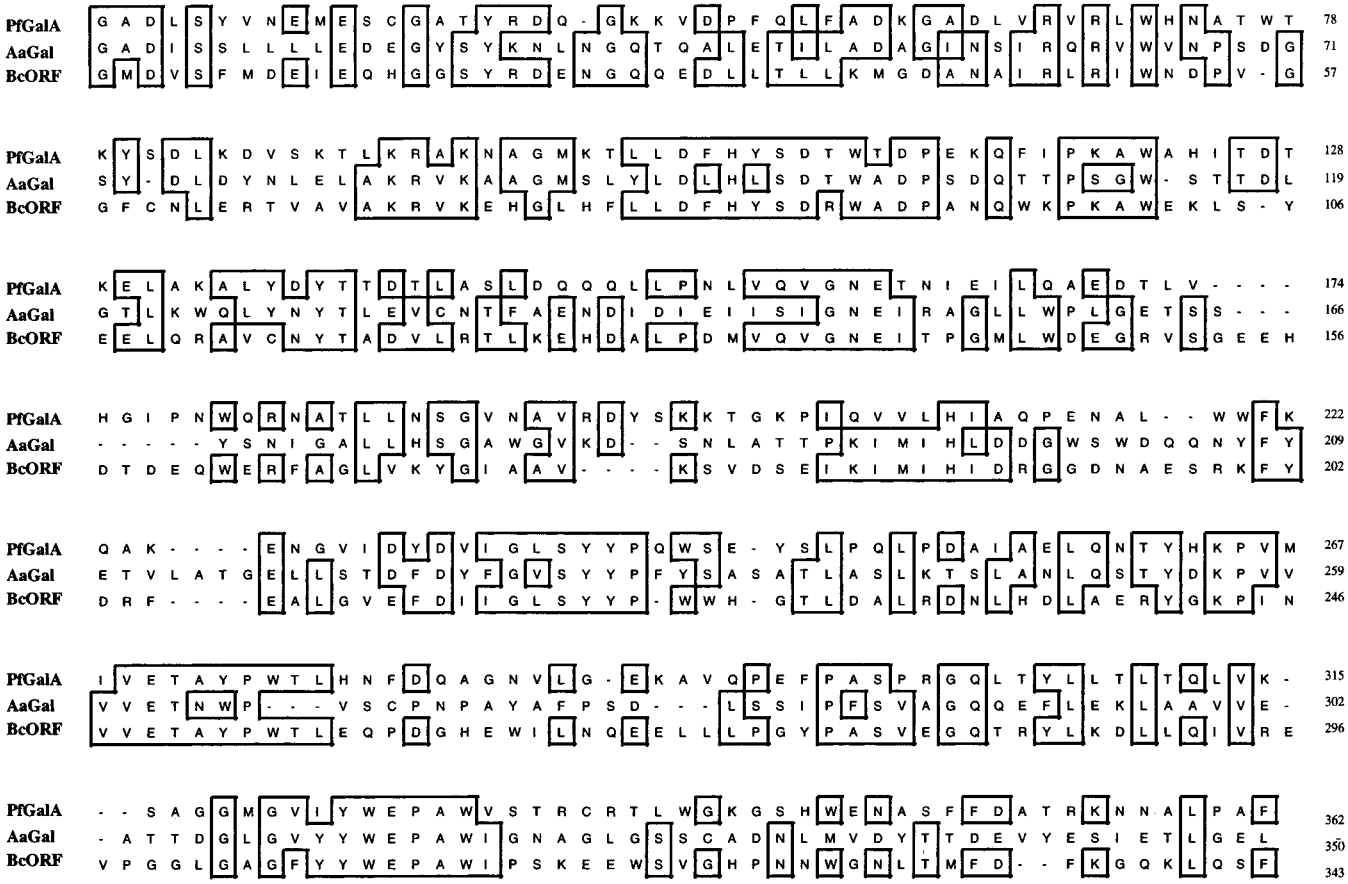

FIGURE 4: Alignment of *Pseudomonas* GalA (PsGalA), *A. cuvatus* galactanase (AcGalA), and a *Bacillus* ORF (BcORF) of unknown function. Amino acids that are conserved in the three sequences are boxed, and the position of the amino acids, at the end of the lines, in the respective primary sequence is indicated. The SWISS-PROT accession numbers of the *Aspergillus* and *Bacillus* and *Pseudomonas* sequences are P48842, P48843, and P48841, respectively.

protein, which was then purified by metal ion affinity chromatography. GalA was released from the fusion protein by treatment with thrombin and purified by removal of the released thioredoxin polypeptide using the metal ion affinity matrix. Figure 5 demonstrates that the galactanase was purified to apparent homogeneity, as judged by SDS/PAGE.

Analysis of the substrate specificity of GalA showed that the enzyme hydrolyzed both lupin and potato β1,4-galactan at similar rates, Azo- and AZCL-galactan, but displayed no catalytic activity against the other plant structural polysaccharides tested, which included mannan, cellulose, xylan, and arabinan (data not shown). Although the enzyme cleaved 2,4-DNPG$_2$, it did not attack 4-nitrophenyl-β-galactoside, xylopyranoside, glucopyranoside, or cellobioside (data not shown). To investigate the mode of action of GalA, the enzyme was incubated with lupin galactan, and the products generated were analyzed by HPLC. Initially a mixture of oligosaccharides were released, but as the reaction proceeded the oligosaccharides became progressively smaller. At the end of the reaction, galactose, galactobiose, and galactotriose were the major products (Figure 6). The release of a mixture of oligosaccahrides by GalA is consistent with a predominantly endo-mode of action.

The sensitivity of plant cell wall hydrolases to extremes of pH and temperature varies depending on their microbial origin. To investigate these properties of GalA, the activity of the enzyme at different temperatures and pH was assessed. The data (Figure 7) showed that the enzyme had pH and temperature optima of 7.5 and 60 °C, respectively. These

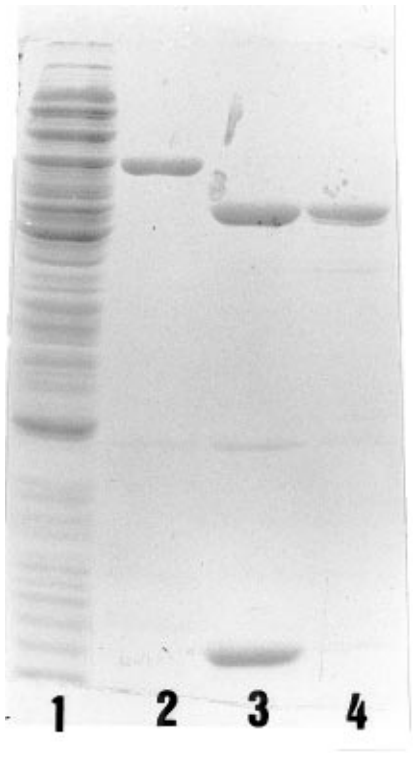

FIGURE 5: SDS/PAGE of purified GalA. SDS/PAGE of cell-free extract derived from *E. coli* harboring pBG3 (lane 1) the thioredoxin-GalA fusion protein purified by Talon affinity chromatography (lane 2), the fusion protein cleaved with thrombin (lane 3), and GalA purified from thioredoxin by rechromatographing on the Talon column (lane 4).

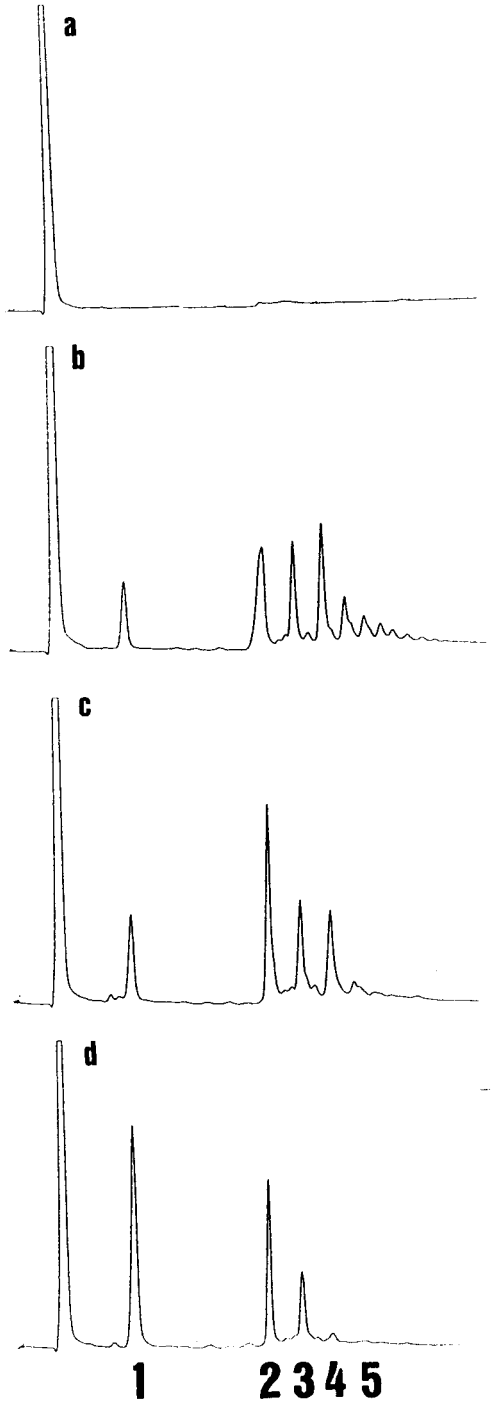

FIGURE 6: HPLC analysis of the products generated from galactan by GalA. Galactan was incubated with GalA for 0 (a), 5 (b), 15 (c), and 240 min (d) as described in Materials and Methods, and the oligosaccharides generated were analyzed by HPLC. The elution time of galactose (1), galactobiose (2), galactotriose (3), galactotetraose (4), and galactopentaose (5) are indicated.

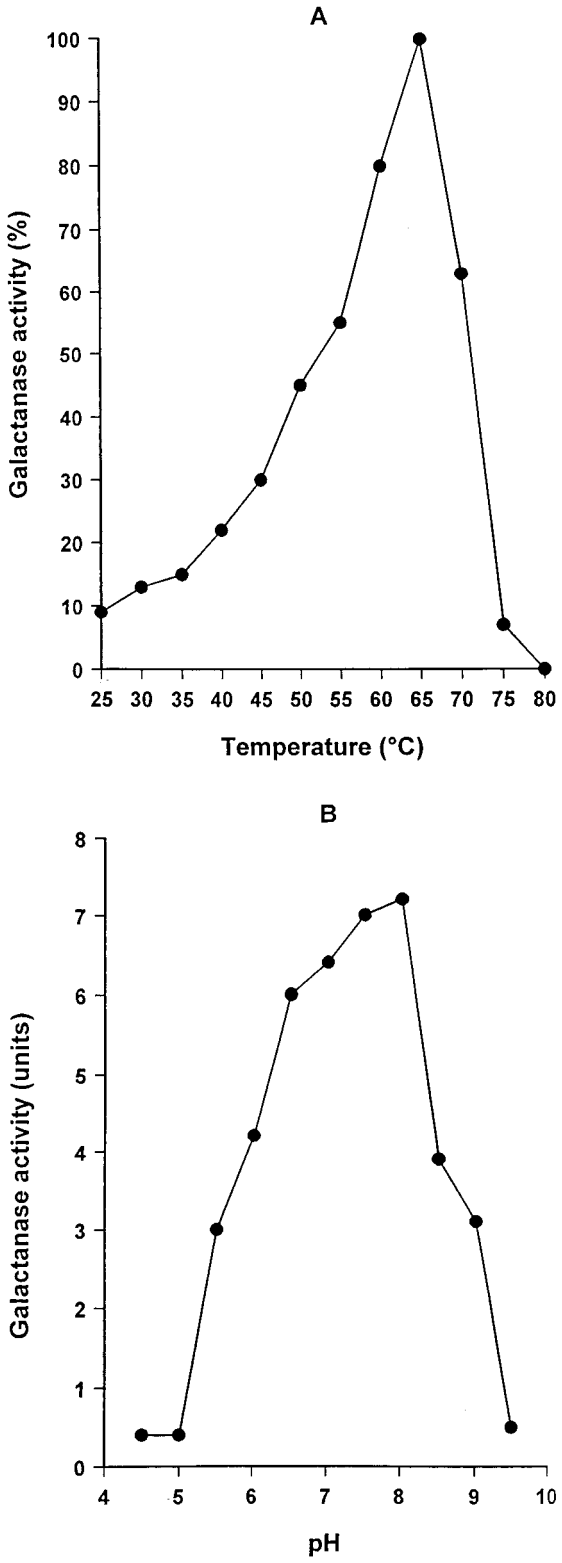

FIGURE 7: Temperature and pH activity profiles of GalA. The activity of GalA at different temperatures (panel A) and pH (panel B) were determined using potato galactan as the substrate. In both experiments, GalA was assayed directly at the pH values and temperatures stated for up to 5 min.

parameters are consistent with the growth characteristics of *P. fluorescens* subsp. *cellulosa*, a mesophilic bacterium that displays maximum growth rate at neutral pH.

Recent studies have shown that many plant cell wall hydrolases exhibit remarkable resistance to proteolytic attack (*32*, *33*). To evaluate whether GalA exhibited this property, the galactanase and a proteinase-resistant fungal xylanase (xylanase A from *Neocallimastix patriciarum*; *34*) were incubated with trypsin, and the loss of catalytic activity of the respective enzymes was monitored with time. The data clearly showed that GalA was sensitive to proteinase inactivation; there was no reduction in xylanase activity over the 1-h hour incubation period, while GalA lost 50% of its activity after 7 min (data not shown).

Stereochemical Course of 2,4-DNPG$_2$ Hydrolysis. To investigate the catalytic mechanism of GalA, the purified

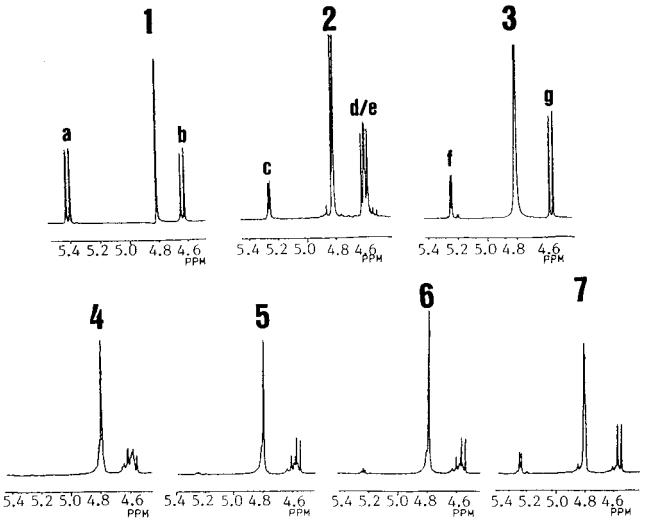

FIGURE 8: ^{1}H-NMR analysis of 2,4-DNPG$_2$ hydrolysis by GalA. ^{1}H-NMR spectra of 2,4-DNPG$_2$, galactobiose, and galactose are displayed in panels 1, 2, and 3, respectively. The ^{1}H-NMR spectrum of 2,4-DNPG$_2$ incubated with GalA for 2 min (panel 4), 30 min (panel 5), 240 min (panel 6), and 48 h (panel 7) are shown. The H-1β (a) and H-1′β (b) signals of 2,4-DNPG$_2$; the H-1α (c), H-1β (d), and H-1′β (e) signals of galactobiose; and the H-1α (f) and H-1β (g) signals of galactose are indicated. The signal at δ 4.8, present in each NMR spectrum, is residual HOD.

Table 1: ^{1}H-NMR of Galactose, Galactobiose, and 2,4-DNPG$_2$

	chemical shifts (ppm) and coupling constants (Hz)[a]		
compound	H-1α	H-1β	H-1′β
2,4-DNPG$_2$		5.40 (7.6)	4.62 (7.6)
galactobiose	5.24 (3.6)	4.59 (7.8)	4.57 (7.5)
galactose	5.23 (3.5)	4.55 (7.8)	

[a] Chemical shifts and coupling constants (in parentheses) for the anomeric hydrogen doublet signals in the ^{1}H-NMR spectra of 2,4-DNPG$_2$ and the α and β anomers of galactobiose and galactose.

enzyme was incubated with 2,4-DNPG$_2$ in D_2O, and the stereochemical course of the reaction was monitored by ^{1}H-NMR. The data, presented in Figure 8 and Table 1, show the chemical shifts and coupling constants of the H-1β and H-1α signals of galactose and galactobiose, the H-1β signal of 2,4-DNPG$_2$, and the H-1′β signals of galactobiose and 2,4-DNPG$_2$. The ratios of the α:β signals of H-1 (3:5) were identical for galactose and galactobiose. To initiate the reaction, 10 unit of GalA was added to 17.5 μmol of 2,4-DNPG$_2$ in 0.5 mL of D_2O in a 5-mm NMR tube at 23 °C. Within 2 min of the start of the reaction, the glycosidic bond between 2,4-DNP and galactobiose had been completely cleaved, as evidenced by the loss of the signal at 5.40 ppm. The spectrum of the 2-min reaction revealed several peaks in the region of 4.55−4.59 ppm, which corresponded to H-1′β and H-1β of galactobiose and H-1β of galactose, suggesting that some of the galactobiose generated had been hydrolyzed to β-galactose. There was no peak in the region δ 5.23−5.24, corresponding to the H-1α of either galactobiose or galactose, in the 2-min spectrum. After 30 min, the anomeric doublet at δ 4.55 of β-galactose had become prominent in this region of the spectrum, and there were very small peaks at δ 5.24 and 5.23, indicating that trace amounts of the α forms of galactobiose and galactose were present. At 240 min, the anomeric doublets at δ 5.24 and 5.23, corresponding to H-1α of galactose and galactobiose had clearly begun to appear. Integration of the H-1β and H-1α signals in the 240-min spectrum showed that the ratio of the α:β forms of galactose and galactobiose was 0.7:6.7. Eventually, after 2 days, the NMR spectrum of the reaction revealed only the doublets correponding to the α and β forms of galactose. Integration of the H-1α and H1-β signals of galactose in the 2-day spectrum showed that the ratio of the α and β forms of the sugar was 1.1:2.6. The NMR spectrum of 2,4-DNPG$_2$, incubated in the absence of GalA for 2 days, indicated that molecule was not hydrolyzed in the absence of GalA (data not shown). These sequence of events clearly demonstrate the rapid enzyme cleavage of 2,4-DNPG$_2$ to the β form of galactobiose, which is then slowly cleaved to the β form of galactose. The much slower appearance of the α forms of galactobiose and galactose is due to mutarotation of the intitally formed β-galactobiose and β-galactose.

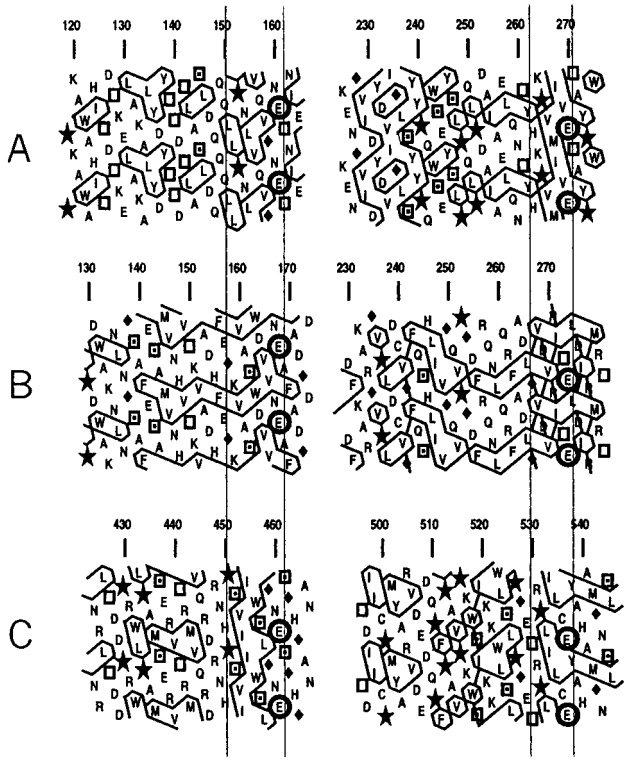

FIGURE 9: HCA comparison of GalA with other members of clan GH-A. Partial HCA plots of (A) galactanase of *P. fluorescens* subsp. *cellulosa*, (B) β-glycanase Cex of *Cellulomonas fimi* and (C) β-galactosidase Z of *E. coli*. The catalytic residues have been circled. The acid−base and nucleophilic residues appear on the left and right partial plots, respectively. Vertical bars delineate the boundaries of the β-strands carrying each catalytic residue. Proline (★), glycine (♦), serine (⊡), and threonine (□) are represented respectively by the symbols to facilitate visual inspection of the plots.

HCA and the Identification of Potential Catalytic Residues of GalA. Recently, HCA and X-ray crystallographic data of plant cell wall hydrolases have established that several glycosyl hydrolase families have evolved from a common ancestral sequence and constitute a superfamily called clan GH-A (*4*, *35*). The relatedness of family 53 to clan GH-A was established by HCA, suggesting that GalA, in common with other enzymes in this clan, has a $(\alpha/\beta)_8$ structure. The conserved motifs around the catalytic residues of glycosyl hydrolases of clan GH-A were found also to occur in family 53 and allowed the prediction of the two catalytic residues of the galactanase, E161 and E270, and their respective functions (acid−base or nucleophile, respectively; Figure 9).

Table 2: Kinetic Parameters of Wild-Type and Mutant Forms of GalA

	galactan (lupin)	Azo-galactan	2,4-DNPG2	
enzyme	k_{cat}[a]	k_{cat}	k_{cat}	K_m[b]
wild-type	681	326	32	2.3
E161A	ND[c]	ND	0.06	0.004
E270A	ND	ND	ND	*d*

[a] k_{cat} is expressed as moles of product produced per mole of enzyme per second. [b] K_m was in mM. [c] ND, no activity detected. [d] K_m could not be determined as no activity was detected.

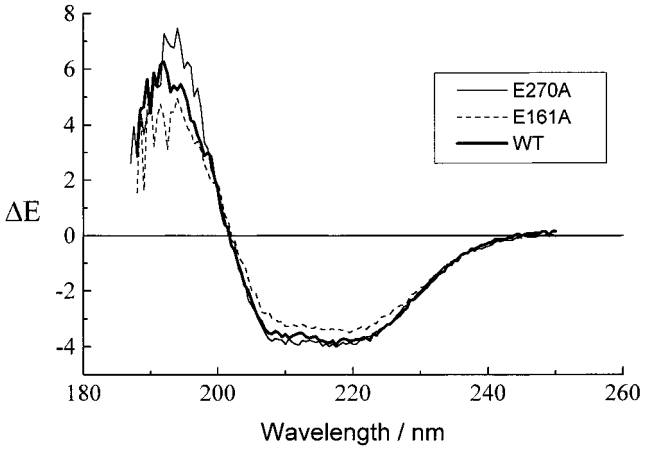

FIGURE 10: CD spectra of wild-type and mutant forms of GalA. Spectra were generated from 20 μM protein solutions in a 0.2 mm path length cell. Scans (10 for each enzyme) were accumulated between 185 and 250 nm. Each spectrum was corrected by subtraction of a comparable blank. The abscissa is represented in units of $\Delta\epsilon$ (M^{-1} cm^{-1}) using the molar concentration of amino acids present. Spectra were not smoothed.

Site-Directed Mutagenesis of Putative Catalytic Residues. To investigate whether E161 and E270 are the key catalytic residues of GalA, site-directed mutagenesis was employed to generate E161A and E270A mutants of the galactanase. The two derivatives of GalA were purified to homogeneity, as judged by SDS/PAGE (data not shown), and the kinetic properties of the enzymes were evaluated. The data (Table 2) showed that replacing E270 with alanine generated an enzyme that was essentially inactive against the highly polymeric substrates (galactan and Azo-galactan) and 2,4-$DNPG_2$. In contrast, E161A did display some activity against the aryl-β-glycoside, although the k_{cat} and K_m of the enzyme were lower than for native GalA activity against the same substrate. E161A did not display activity against galactan or Azo-galactan. To assess the influence of the mutations on the gross structure of GalA, E161A and E270A were subjected to CD spectroscopy. The far-UV CD spectra of wild-type and mutant enzymes are shown in Figure 10. The data reveal that all the proteins share a similar secondary structure content and that the point mutations do not result in significant changes in folding, indicating that E161 and E270 do not play a critical structural role in GalA. Collectively, these data support the view that E161 and E270 constitute the key catalytic residues of the galactanase.

To evaluate whether the decrease in K_m was due to a reduction in k_3, the rate-limiting step of 2,4-$DNPG_2$ hydrolysis by E161A and the wild-type enzyme was determined by analyzing the pre-steady-state kinetics of this reaction. Reaction rates were measured at five substrate concentrations that were greater than the enzyme concentration by a factor >20. Even at the highest concentration of substrate, the initial rate of 2,4-DNP release continued throughout the course of the reaction (10% loss of substrate) when using wild-type enzyme. In contrast, at each of the substrate concentrations tested in reactions containing E161A, there was an initial rapid release of 2,4-DNP (k_{obs}) which then decayed to a slow steady-state rate (k_3) of 0.07 s^{-1}, which was similar to the separately determined steady-state k_{cat} for E161A. The k_{obs} values were fitted into the following equation, which is valid when $k_2 \gg k_3$ (*36*):

$$k_{obs} = \frac{k_2[S]}{K_S + [S]}$$

to derive k_2 and K_S, which were 24 s^{-1} and 3.4 mM, respectively; the K_S was in reasonable agreement with the K_m of the wild-type enzyme. The amplitude of the burst of 2,4-DNP release with E161A was equivalent to 0.7 mol/mol protein (at substrate and enzyme concentrations of 17 μM and and 730 nM). The size of the burst doubled when the enzyme concentration was doubled.

DISCUSSION

Data presented in this paper provide the first description of galactan hydrolysis by *P. fluorescens* subsp. *cellulosa*, providing further support for the view that the bacterium can utilize a wide range of plant structural polysaccharides. The galactanase was induced by galactan, although not by the other major structural polysaccharides, and was subject to catabolite repression. In contrast, *Pseudomonas* cellulases and xylanases are induced by both cellulose and xylan. The differences in the regulation of these enzymes could be a consequence of the origin of the polysaccharides. Virtually all plant cell walls contain both cellulose and xylan, thus either polysaccharide could signal the presence of plant cell wall material and hence act as an inducer of the major microbial plant cell wall polysaccharidases. Galactan, however, is not present in all plant material and is generally loosely associated with other plant structural polysaccharides (*11*). It is apparent, therefore, that as galactan is not always a good indicator of the presence of other plant structural polysaccharides, the expression of galactanases is not coordinated with the synthesis of cellulases and xylanases.

Although *P. fluorescens* contains large multigene families encoding cellulases, xylanases, and mannanases, data presented in this paper and in a previous study (*29*) indicate that the bacterium has single copy of both a galactanase and arabinanase gene. It is possible that this reflects the accessibility of the respective polysaccharides to enzymic attack. Galactan and arabinan are loosely associated with the cell wall and are thus more susceptible to enzymic attack. However, as cellulose, mannan, and xylan are integral components of the plant cell wall, a repertoire of enzymes that recognize different chemical and physical forms of these molecules may be required to elicit efficient hydrolysis.

All the *P. fluorescens* cellulases and xylanases analyzed to date are modular enzymes that contain at least one CBD (*6*−*8*, *37*). We have hypothesized that CBDs facilitate the binding of a range of plant cell wall hydrolases onto the plant cell wall, promoting both enzyme−substrate and enzyme−enzyme interactions that improve the efficiency of catalysis (*5*). The absence of a CBD in GalA could reflect the loose

association of galactan with the plant cell wall, making it more accessible to enzymic attack than integral polysaccharides such as cellulose and xylan. It is interesting to note that arabinan, another plant structural polysaccharide that is not closely associated with the plant cell wall, is also hydrolyzed by a *P. fluorescens* enzyme, ArbA, that does not contain a CBD (*29*).

It is now widely accepted that the mechanism of bond cleavage is conserved in enzymes from the same glycosyl hydrolase family. Thus, based on the mechanism of action of GalA, we propose that all enzymes in family 53 cleave galactan via a double displacement mechanism. The data are also consistent with the assignment of GalA to the GH-A clan, as enzymes in this superfamily also have a double displacement mode of action.

Glycosyl hydrolases cleave glycosidic bonds by a general acid−base mechanism that is mediated by two carboxylic acid residues, one functioning as the nucleophile and the other as the acid−base. HCA predictions and site-directed mutagenesis studies indicate that E161 and E270 constituted the key catalytic residues of GalA. To evaluate which of the two residues functioned as the nucleophile and which was the acid−base, the activity of wild-type and mutant forms of GalA against the substrate 2,4-DNPG_2 was studied. In a retaining glycosyl hydrolase, the nucleophilic residue attacks the anomeric carbon of the substrate (k_2) forming an glycosyl−enzyme intermediate. If the nucleophile has been inactivated via mutagenesis, then no hydrolysis of the substrate would be observed, even when using 2,4-DNPG_2, which has a good leaving group (pK_a of 2,4-DNP is 3.96). Thus, the lack of activity of E270A against either galactan or 2,4-DNPG_2 is consistent with the nucleophilic role of E270 in catalysis. In glycosidases that exhibit a double displacement mechanism, the acid−base residue protonates the glycosidic oxygen promoting bond cleavage and catalyzes hydrolysis of the glycosyl−enzyme intermediate by abstracting a proton from water. Removal of the acid−base residue would decrease the deglycosylation rate (k_3) of the enzyme against 2,4-DNPG_2, resulting in the accumulation of the glycosyl−enzyme intermediate and a decrease in the K_m (*38*). Against substrates with poor leaving groups, the acid−base mutant would be unable to catalyze the initial bond cleavage, as no protonation of the glycosidic oxygen would occur. The substantial reduction on K_m and k_{cat} of E161A against 2,4-DNPG_2 and the lack of activity of the mutant against galactan are entirely consistent with the proposed role of E161 as the acid−base catalyst.

Further support for the role of E161 as the acid−base residue was obtained by studying the pre-steady-state kinetics of 2,4-DNPG_2 hydrolysis by E161A. The pre-steady-state burst of 2,4-DNP release, observed in reactions catalyzed by E161A, was not evident when using wild-type GalA. Although it is formerly possible that the wild-type enzyme does generate a pre-steady-state burst of 2,4-DNP release, which is too rapid for the stopped-flow apparatus to detect, this event is very unlikely for the following reasons: (i) the K_m of the reaction catalyzed by native GalA is relatively high (mM range), indicating that a glycosyl−enzyme intermediate does not accumulate; (ii) the k_2 value for E161A was similar to the k_{cat} for the wild-type enzyme, which is consistent with the view that k_2 is the rate-limiting step in catalysis mediated by native GalA. Collectively, the pre-steady-state kinetic data indicate that although E161A and the wild-type enzyme catalyze the glycosylation reaction at similar rates, the mutant form of GalA catalyzes the deglycosylation step very slowly, while native GalA hydrolyzes the glycosyl−enzyme intermediate more rapidly than the glycosylation step; hence, there was no obvious pre-steady-state burst of 2,4-DNP release. The switch in the rate-limiting step of the reaction from glycosylation with the wild-type enzyme to deglycosylation when using E161A is entirely consistent with the proposed role for E161 as the acid−base catalyst, as this catalytic residue is only required in the deglycosylation step when using a substrate that does not require protonation of the glycosidic oxygen for initial bond cleavage to occur. It is interesting to note that E161A still catalyzes deglycosylation, although at a greatly reduced rate. A similar phenomenon has been observed for other retaining glycosyl hydrolases in which the acid−base residue has been mutated (*23*, *38*). Presumably, this reflects the contribution made by other active site residues in catalyzing deglycosylation.

To conclude, this paper provides the first report of the primary structure and catalytic mechanism of a prokaryotic galactanase. It is apparent that GalA, which is closely related to a fungal galactanase, catalyzes glycosidic bond cleavage via a double displacement mechanism, and HCA predicts that the enzyme belongs to the GH-A clan of $(\alpha/\beta)_8$ barrel glycosyl hydrolases in which E161 and E270 are the acid−base and nucleophile catalytic residues, respectively. Kinetic analysis of derivatives of GalA in which E161 and E270 have been mutated provide further support for their predicted role in catalysis. Although kinetic data alone do not distinguish between the pivotal catalytic residues and those amino acids that play an important role in the function of the catalytic residues, the assignment of E161 and E270 as the acid−base and nucleophilic catalysts, respectively, is supported by HCA of the enzyme.

ACKNOWLEDGMENT

The authors would like to thank Judy Laurie for her excellent technical assistance.

REFERENCES

1. Withers, S. G. (1995) in *Progress in Biotechnology Volume 10, Carbohydrate Bioengineering* (Petersen, S. B., Svensson, B., Pedersen, S., Eds.) pp 97−112, Elsevier, Amsterdam, The Netherlands.
2. McCarter, J. D., and Withers, S. G. (1994) *Curr. Opin. Struct. Biol. 4*, 885−892.
3. Henrissat, B., and Davies, G. (1995) *Structure 3*, 853−859.
4. Henrissat, B., Callebaut, I., Fabrega, S., Lehn, P., Mornon, J.-P., and Davies, G. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 7090−7094.
5. Ferreira, L. M. A., Wood, T. M., Williamson, G., Faulds, C., Hazlewood, G. P., Black, G. W., and Gilbert, H. J. (1993) *Biochem. J. 294*, 349−355.
6. Millward-Sadler, S. J., Davidson, K., Hazlewood, G. P., Black, G. W., Gilbert, H. J., and Clarke, J. H. (1995) *Biochem. J. 312*, 39−48.
7. Gilbert, H. J., Hall, J., Hazlewood, G. P., and Ferreira, L. M. A. (1990) *Mol. Microbiol. 4*, 759−767.
8. Kellett, L. E., Poole, D. M., Ferreira, L. M. A., Durrant, A. J., Hazlewood, G. P., and Gilbert, H. J. (1990) *Biochem. J. 272*, 369−376.
9. Tomme, P., Warren, R. A. J., and Gilkes, N. R. (1995) *Adv. Microbiol. Physiol. 37*, 1−81.
10. Selvendran, R. R. (1983) in *The Chemistry of Plant Cell Walls, Dietary Fibres*; Applied Science Publishers, NewYork.

11. Hwang, Y., Pyun, Y. R., and Kokini, J. L. (1993) *Food Hydrocolloids 7*, 39−53.
12. Emi, S., and Yamamoto, T. (1972) *Agric. Biol. Chem. 36*, 1945−1954.
13. Araujo, A., and Ward, O. P. (1990) *J. Ind. Microbiol. 6*, 171−178.
14. Christgau, S., Sandal, T., Kofod, L. V., and Dalboge, H. (1995) *Curr. Genet. 27*, 135−141.
15. Gilbert, H. J., Jenkins, G., Sullivan, D. A., and Hall, J. (1987) *Mol. Gen. Genet. 210*, 551−556.
16. Gilbert, H. J., Sullivan, D. A., Jenkins, G., Kellett, L. E., Minton, N. P., and Hall, J. (1988) *J. Gen. Microbiol. 134*, 551−556.
17. Norrander, J., Kempt, T., and Messing, J. (1983) *Gene 26*, 101−106.
18. Studier, F. W., and Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113−130.
19. Chambers, S. P., Prior, S. E., Barstow, D. A., and Minton, N. P. (1988) *Gene 68*, 139−149.
20. Braithwaite, K. L., Black, G. W., Hazlewood, G. P., Ali, B. R. S., and Gilbert, H. J. (1995) *Biochem. J. 305*, 1005−1010.
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual* (Ford, N., Ed.) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
22. Staden, R. (1980) *Nucleic Acid. Res. 16*, 3673−3694.
23. Bolam, D. N., Hughes, N., Virden, R., Lakey, J. H., Hazlewood, G. P., Henrissat, B., and Gilbert, H. J. (1996) *Biochemistry 35*, 16195−16204.
24. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
25. Miller, G. L. (1959) *Anal. Chem. 31*, 426−428.
26. Charnock, S. J., Lakey, J. H., Virden, R., Hughes, N., Sinnott, M. L., Hazlewood, G. P., Pickersgill, R., and Gilbert, H. J. (1997) *J. Biol. Chem. 272*, 2942− 2951.
27. Sedmak, J. J., and Grossberg, J. R. (1977) *Anal. Biochem. 79*, 544−552.
28. Laemmli, U. K. (1970) *Nature 227*, 680−685.
29. McKie, V. A., Black, G. W., Millward-Sadler, S. J., Hazlewood, G. P., Laurie, J. I., and Gilbert, H. J. (1997) *Biochem. J. 323*, 547−555.
30. Perlman, D., and Halvorson, H. O. (1983) *J. Mol. Biol. 167*, 391−409.
31. Henrissat, B., and Bairoch, A. (1996) *Biochem. J. 316*, 695−696.
32. Hall, J., Ali, S., Surani, M. A., Hazlewood, G. P., Clark, A. J., Simons, J. P., Hirst, B. H., and Gilbert, H. J. (1993) *Bio/Technology 11*, 376−379.
33. Fontes, C. M. G. A., Hall, J., Hirst, B. H., Hazlewood, G. P., and Gilbert, H. J. (1995) *Appl. Microbiol. Biotechnol. 43*, 52−57.
34. Gilbert, H. J., Hazlewood, G. P., Laurie, J. I., Orpin, C. G., and Xue, G. P. (1992) *Mol. Microbiol. 6*, 2065−2072.
35. Jenkins, J., Lo Leggio, L., Harris, G., and Pickersgill, R. (1995) *FEBS Lett. 362*, 281−285.
36. Gutfreund, H., and Strutevant, J. M. *Biochem. J. 63*, 656−661.
37. Ferreira, L. M. A., Durrant, A. J., Hall, J., Hazlewood, G. P., and Gilbert, H. J. (1990) *Biochem. J. 269*, 261−264.
38. MacLeod, A. M., Lindhorst, T., Withers, S. G., and Warren, R. A. J. (1994) *Biochemistry 33*, 6371−6376.

BI9712394