A non-modular endo- β -1,4-mannanase from *Pseudomonas fluorescens* subspecies *cellulosa*

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Pseudomonas fluorescens subsp. *cellulosa* when cultured in the presence of carob galactomannan degraded the polysaccharide. To isolate gene(s) from *P. fluorescens* subsp. *cellulosa* encoding endo- β -1,4-mannanase (mannanase) activity, a genomic library of *Pseudomonas* DNA, constructed in λ ZAPII, was screened for mannanase-expressing clones using the dye-labelled substrate, azo-carob galactomannan. The nucleotide sequence of the pseudomonad insert from a mannanase-positive clone revealed a single open reading frame of 1257 bp encoding a protein of M_r 46 938. The deduced N-terminal sequence of the putative polypeptide conformed to a typical prokaryotic signal peptide. Truncated derivatives of the mannanase, lacking 54 and 16 residues from the N- and C-terminus respectively of the mature form of the enzyme, did not exhibit catalytic activity. Inspection of the primary structure of the mannanase did not reveal any

obvious linker sequences or protein motifs characteristic of the non-catalytic domains located in other *Pseudomonas* plant cell wall hydrolases. These data indicate that the mannanase is non-modular, comprising a single catalytic domain. Comparison of the mannanase sequence with those in the SWISSPROT database revealed greatest sequence homology with the mannanase from *Bacillus* sp. Thus the *Pseudomonas* enzyme belongs to glycosyl hydrolase Family 26, a family containing mannanases and endoglucanases. Analysis of the substrate specificity of the mannanase showed that the enzyme hydrolysed mannan and galactomannan, but displayed little activity towards other polysaccharides located in the plant cell wall. The enzyme had a pH optimum of approx. 7.0, was resistant to proteolysis and had an M_r of 46 000 when expressed by *Escherichia coli*.

INTRODUCTION

The plant cell wall is hydrolysed by a wide variety of endo- and exo-acting β -1,4-glycosidases, in addition to accessory enzymes that remove the side chains from hemicelluloses such as xylan (Gilbert and Hazlewood, 1993). Most studies on plant cell wall hydrolases have focused to date on enzymes that hydrolyse cellulose and xylan, the primary constituent of hemicellulose in grasses. Enzymes that hydrolyse mannan have been largely neglected, even though it is an abundant hemicellulose, especially in softwoods, seeds of leguminous plants and in carob beans. Mannan can exist as a linear polymer of β -1,4-mannose units or as a heterogeneous polymer of β -1,4-linked mannose and glucose units (glucomannan). The mannose units in mannan and glucomannan are often substituted with galactose and *O*-acetyl groups (Puls and Schuseil, 1993). The mannan backbone is hydrolysed by endo- β -1,4-mannanases (mannanases) to mannobiose and small oligosaccharides, which are cleaved by β -mannosidases to release mannose. To date, the molecular structures of three mannanases have been analysed: from *Streptomyces lividans* (Arcand et al., 1993), *Bacillus* sp. (Akino et al., 1989) and *Caldocellum saccharolyticum* (Gibbs et al., 1992). The three enzymes belong to glycosyl hydrolase Families 26 and 5 (Henrissat, 1991), both of which are comprised of endoglucanases. The *Streptomyces* and *Bacillus* mannanases consist of a single catalytic domain, whereas the *C. saccharolyticum* enzyme contains discrete mannanase and endoglucanase catalytic domains, in addition to cellulose-binding-domain (CBD) homologues.

Recent studies in our laboratories have focused on the plant cell wall hydrolases of *Pseudomonas fluorescens* subsp. *cellulosa*. The endoglucanases, xylanases, arabinofuranosidase, acetyl xylan esterase and cellobextrinase characterized to date all have a modular structure consisting of a catalytic domain, linked by serine-rich sequences to a non-catalytic CBD (Hazlewood and Gilbert, 1992). To evaluate whether the location of CBDs is a universal phenomenon of *Pseudomonas* plant cell wall hydrolases, and to increase our understanding of the relatively little studied enzymes that hydrolyse the important hemicellulose mannan, we have initiated studies designed to analyse the structure and function of microbial mannanases. In this report we demonstrate that *P. fluorescens* subsp. *cellulosa* is capable of degrading mannan, and we describe the biochemical properties and molecular architecture of a *Pseudomonas* mannanase. The data show that the enzyme exhibits narrow substrate specificity and, unlike other *Pseudomonas* plant cell wall hydrolases, does not contain a CBD. The enzyme exhibits 19% sequence identity with the *Bacillus* mannanase, placing it in glycosyl hydrolase Family 26. The relationship between the structure and function of the enzyme is discussed.

MATERIALS AND METHODS

Microbial strains, vectors and media

P. fluorescens subsp. *cellulosa* and *Escherichia coli* were cultured at 37 °C in Luria broth. Luria broth was supplemented with ampicillin (100 μ g/ml) and 5-bromo-4-chloro-3-indolyl β -D-galactoside (2 μ g/ml) to select for *E. coli* transformants and

Abbreviations used: MANA, mannanase A; IPTG, isopropyl thio- β -D-galactopyranoside; CBD, cellulose-binding domain; 18MANA, MANA lacking its 18 N-terminal residues; MBP, maltose-binding protein.

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The nucleotide sequence of *manA* will appear in the EMBL/GenBank/DBJ Nucleotide Sequence Databases under the accession number X82179.

recombinants respectively. Azo-carob galactomannan was added to solid media at a final concentration of 0.2% to select for mannanase-positive micro-organisms. To induce expression of genes subject to *lacO* control, isopropyl thio- β -D-galactopyranoside (IPTG) was added to culture media at a final concentration of 0.1 mM and induction was allowed to proceed for up to 16 h. λ phage were cultured in NZY medium seeded with the *E. coli* indicator strain XL1-Blue. The vectors employed in this study were λ ZAPII (Stratagene), pBluescript SK⁻ (Stratagene), pET21a (Novagen), pNM52 (Gilbert et al., 1986), pMAL-c2 (New England Biolabs), pMTL22p and pMTL23p (Chambers et al., 1988).

Construction and screening of gene library

P. fluorescens subsp. *cellulosa* DNA, prepared as described previously (Gilbert et al., 1987), was partially digested with *Sau*3A, and DNA in the size range 3–10 kb was purified by agarose-gel electrophoresis and ligated into *Xho*I-cleaved λ ZAPII using the partial fill-in technique described in the supplier's protocol (Stratagene). The DNA was packaged *in vitro* and infected into *E. coli* XL1-Blue to generate approx. 10^6 recombinant phage. To screen the library for mannanase-producing clones, phage were plated out on NZY top agarose supplemented with azo-carob galactomannan (0.2%). After incubation for 16 h at 37 °C mannanase-synthesizing phage were evident by the appearance of clear haloes against a blue background. *Pseudomonas* DNA from mannanase-positive phage was excised and recircularized to form phagemid (pBluescriptSK⁻) as described previously (Black et al., 1994).

General recombinant DNA methodology

Agarose-gel electrophoresis, Southern hybridization, transformation of *E. coli* and the use of restriction enzymes and T4 DNA ligase were as described by Gilbert et al. (1987). Plasmid DNA was prepared using Qiagen columns. To sequence *manA*, nested deletions of appropriate restriction fragments were generated using the exonuclease III/S1-nuclease method. Truncated DNA was sequenced by the dideoxy chain termination method of Sanger et al. (1980) using Sequenase version 2.0. Preparation and denaturation of plasmid DNA before sequencing was performed as described by Black et al. (1994). Sequences were compiled and ordered using the computer programs described by Staden (1980). The complete *manA* sequence was determined in both strands. To clone the *manA* gene into pET21a, *Bam*HI and *Xho*I restriction sites were introduced 5' and 3' respectively of *manA* by PCR. The primers used to amplify the sequence between map positions -14 and +1266 were 5'-CGCGGATCCATCCCGA-GAGGACAACAATG-3' and 5'-CCGCTCGAGTAACTATC-AGGGTCGGACGC-3' respectively, and the conditions for PCR were as follows. The reaction buffer consisted of 10 mM Tris/HCl, pH 8.3, containing 50 mM KCl, four dNTPs (each 200 μ M), 3 mM MgCl₂, 1 unit of *Taq* polymerase, 50 pg of primer and 1 ng of DNA in a reaction volume of 100 μ l. The DNA was initially denatured at 96 °C for 6 min, before the addition of the enzyme, and subsequently the reaction was allowed to proceed through 30 cycles of 1 min at 96 °C, 1 min at 55 °C and 2 min at 72 °C. The amplified DNA was cloned directly into pCRII (Invitrogen) and then subcloned into pET21a.

Enzyme and protein assays

Cell-free extracts from recombinant *E. coli* strains were prepared by sonication. Standard enzyme assays were performed using 0.2% (final concentration) of the appropriate plant structural

polysaccharide as the substrate in 0.5 ml of 50 mM potassium phosphate/12 mM citric acid buffer, pH 6.5 (PC buffer). The reaction was initiated by the addition of 50 μ l of an appropriate dilution of enzyme, and the assay was allowed to proceed for 10 min at 37 °C. The reaction was terminated by the addition of 0.5 ml of 3,5-dinitrosalicylic acid reagent (Miller, 1959) and heating to 100 °C for 20 min. Measurement of A_{575} was used to quantify the amount of reducing sugar released from the various substrates. One unit of enzyme activity is the amount of enzyme that releases 1 μ mol of reducing sugar/min. at 37 °C.

To identify the products released by mannanase A (MANA), *E. coli* cell-free extracts were incubated with 0.2% of the appropriate mannan or 0.66 mg/ml manno-oligosaccharides, and at regular time intervals the reactions were terminated by heating to 100 °C for 5 min. The reaction products were subjected to h.p.l.c. analysis using a Dionex PA1 column and eluted with 100 mM NaOH containing a 0–75 mM gradient of sodium acetate. The sugars were detected with a pulsed amperometer, and quantified by comparing the response produced by the various test samples with standard amounts of mannose and manno-oligosaccharides up to mannohexaose. There was a linear relationship between the amount of standard subjected to h.p.l.c. and the response of the pulse amperometer. This applied for each standard evaluated.

The effect of MANA on the viscosity of a 2.5% (w/v) solution of carob galactomannan in PC buffer was determined using a Contraves low-shear 30-cuvette rotating-cup viscometer. The capacity of MANA to bind to crystalline cellulose was evaluated as described by Ferreira et al. (1993). NADH oxidoreductase was determined as described by Osborn et al. (1972), glyceraldehyde 3-phosphate dehydrogenase by the method of Suzuki and Harris (1971) and β -galactosidase as reported by Rosenthal (1987).

The size of the recombinant mannanase produced by *E. coli* harbouring *manA* in pKB6 was determined by zymogram analysis after SDS/PAGE of the partially purified enzyme. Cell-free extract was prepared from *E. coli* cells that had been grown to early-exponential phase and induced for 7 h after the addition of IPTG. MANA was quantitatively recovered in the precipitate formed by adding (NH₄)₂SO₄ (0.313 g/ml), and was redissolved in 10 mM Tris/HCl buffer, pH 8.0, and dialysed against 3×1000 vol. of the same buffer. The concentrated fraction containing MANA was subjected to anion-exchange chromatography on DEAE-Trisacryl (26 mm $\times$ 300 mm column), using a 420 ml elution gradient containing from 0 to 400 mM NaCl at a flow rate of 25 ml/h, and thereafter to f.p.l.c. on Mono Q (Pharmacia), using a 1 ml analytical column and a 40 ml gradient containing from 0 to 1 M KCl at a flow rate of 60 ml/h. Fractions containing mannanase activity, eluted at 160 mM KCl, were analysed by SDS/PAGE (Laemmli, 1970) in 10% (w/v) polyacrylamide gels containing 0.2% carob galactomannan. Renaturation of mannanase and zymogram staining were carried out as described by Malburg and Forsberg (1993).

Protein was determined by the method of Sedmak and Grossberg (1977).

Purification of fusion protein

The maltose-binding-protein (MBP)–MANA fusion protein was purified from *E. coli* cells harbouring pKB5 by amylose affinity chromatography as described by Black et al. (1994).

Source of reagents

Manno-oligosaccharides, mannans, arabinan, galactan and wheat and rye arabinoxylan were purchased from Megazyme (Australia) Pty Ltd. IPTG, restriction endonucleases, T4 DNA

ligase and agarose were obtained from Gibco-BRL. The exonuclease III/S1-nuclease nested deletion kit was purchased from Promega. The Sequenase sequencing kit was obtained from United States Biochemicals. Oligonucleotides were made by the Central Molecular Biology Facility at the University of Newcastle upon Tyne. *Taq* polymerase was purchased from Applied Biosystems Instruments. Qiagen plasmid purification columns were from Hybaid Ltd. Radiolabelled dATP was purchased from Amersham International. All other reagents were obtained from Sigma.

RESULTS

P. fluorescens subsp. *cellulosa* mannanase activity

To evaluate whether *P. fluorescens* subsp. *cellulosa* was capable of hydrolysing mannan, the bacterium was cultured on Luria agar supplemented with azo-carob galactomannan. Colonies, which appeared after 48 h, were surrounded by clear haloes against a blue background, indicating that the pseudomonad produces enzymes that hydrolyse galactomannan. The nature of these enzyme(s) was uncertain. It is possible that the bacterium synthesized specific mannanase(s) or enzymes with wide substrate specificities which hydrolysed a range of polysaccharides, including mannan. Previous studies have reported both enzyme species; *Bacillus* sp. and *S. lividans* express mannanases with narrow substrate specificities (Akino et al., 1989; Arcand et al., 1993), whereas *C. saccharolyticum* produces an enzyme that hydrolyses both mannan and cellulosic substrates (Gibbs et al., 1992). By cloning the genes encoding *Pseudomonas* enzymes that hydrolyse mannan into a host, such as *E. coli*, that has no endogenous polysaccharidase activity, the substrate specificity of each mannanase can be unambiguously defined.

Cloning of *manA*

To isolate the mannanase gene(s), a genomic library of *P. fluorescens* subsp. *cellulosa* DNA was constructed in λ ZAPII and screened for clones expressing mannanase activity. One of several

Figure 1 Restriction map of *Pseudomonas* genomic DNA containing *manA*

The location of the cleavage sites for *Apa*I (A), *Eco*RI (R), *Eco*RV (RV), *Hind*III (H), *Kpn*I (K), *Nco*I (N), *Pst*I (P), *Sma*I (S) and *Sst*I (Ss) are shown. In plasmid pKB4 the 5' truncated form of *manA* was cloned into pMTL22p, such that the mannanase gene was in-phase with the vector's *lacZ'*. The plasmid pKB5 was constructed as follows: The 2 kb *Nco*I–*Bam*HI restriction fragment (map position 0–2 kb) from pKB1 was cloned into *Bgl*II–*Nco*I-restricted pMTL22p. The pseudomonad insert was then excised on a partial *Eco*RI restriction fragment and cloned into the *Eco*RI site of pMAL-c2, such that the 5' truncated mannanase gene was in-frame with *malA*. The *Pseudomonas* insert in plasmid pKB6 consisted of a PCR-amplified region of pKB1 containing *manA*, which was cloned into pET21a. The locations of the primers used in the PCR amplification are shown in Figure 5. The solid arrow shows the extent and orientation of *manA*. The catalytic activity of MANA encoded by the recombinant plasmids is shown (+/–).

Figure 2 Southern hybridization of *Pseudomonas* genomic DNA with *manA*

P. fluorescens subsp. *cellulosa* genomic DNA, either uncut (lane 1) or restricted with *Eco*RV (lane 2), *Bam*HI (lane 3) or *Sal*I (lane 4), was subjected to Southern hybridization using the pseudomonad insert from pKB5 as the probe. The location and size (kb) of standard DNA fragments comprising λ DNA digested with *Hind*III are indicated.

mannanase-positive recombinant phage was isolated, purified and the pseudomonad DNA excised into pBluescriptSK[–] using helper phage. *E. coli* cells containing the resultant recombinant plasmid, designated pKB1, synthesized a functional mannanase, defined as mannanase A (MANA). The restriction map of the *Pseudomonas* DNA insert in pKB1 is depicted in Figure 1. To locate the position of the mannanase gene, designated *manA*, various restriction fragments were either deleted from pKB1 or subcloned into other vectors, and the capacity of the recombinant plasmids generated to direct the expression of a functional mannanase was evaluated. The data, presented in Figure 1, show that *manA* was located between the multiple cloning region of pKB1 (position 0) and the *Apa*I restriction site at position 4.25 kb. To establish whether *manA* was a single-copy gene, the 2.0 kb pseudomonad insert from pKB5 was used to probe restricted *Pseudomonas* genomic DNA. The hybridization pattern indicated that *manA* was a single-copy gene (Figure 2), with no homologous sequences located elsewhere in the *Pseudomonas* genome. Cross-hybridization experiments between *manA* and the other mannanase-positive recombinant phage showed that the pseudomonad contains at least three distinct mannanase genes that do not exhibit significant sequence identity.

Characteristics of MANA

The substrate specificity of MANA produced by pKB6 was determined. The enzyme was active against carob galactomannan (9.46 units/mg of protein), locust-bean galactomannan (4.21 units/mg of protein) and ivory-nut mannan (0.96 unit/mg of protein). Hydrolysis of carob galactomannan was associated with a rapid decline in the viscosity of the substrate, indicating that the enzyme was an endo- rather than an exo-acting mannanase (results not shown). No reducing sugar was detected when cell-free extract containing the mannanase was incubated with a range of other polysaccharides including CM-cellulose, Avicel, acid-swollen cellulose, β -glucan, laminarin, β -1,4-galactan, arabinan and arabinoxylans from oat spelts, wheat and rye.

Figure 3 Size of recombinant MANA

Cell-free extract (40 µg of protein) derived from *E. coli* harbouring pKB6 (lane 1) and 1.1 µg of partially purified MANA (lanes 2 and 3) were subjected to SDS/PAGE, and the resultant polyacrylamide gel was either stained with Coomassie Blue (lanes 1 and 2) or subjected to zymogram analysis (lane 3) as described in the Materials and methods section. M_r ($\times 10^{-3}$) of MANA is shown.

These data show that MANA is a mannanase with narrow substrate specificity which does not hydrolyse other polysaccharides.

The high activity against carob and locust-bean galactomannan, compared with ivory-nut mannan, could reflect the more open structure of the substituted mannans. H.p.l.c. analysis of the products generated from ivory-nut mannan and carob galactomannan revealed primarily mannobiose, mannotriose and some mannose. To establish whether MANA exhibited maximum activity against long-chain mannan molecules, the capacity of the enzyme to hydrolyse a range of water-soluble manno-oligosaccharides was evaluated. The relative activity (time taken for 50% cleavage of the substrate) of MANA against mannotriose, mannotetraose, mannopentaose and mannohexaose was 0.25, 0.63, 0.65 and 1 respectively, confirming that MANA preferentially hydrolyses the larger oligosaccharides. After prolonged incubation of MANA with the various oligosaccharides, the major products released were mannose and mannobiose. The ratios of disaccharide/monosaccharide for the substrates mannotriose, mannotetraose, mannopentaose and mannohexaose were 2:3, 4:1, 5.3:4.7 and 6.4:3.6 respectively. No significant activity was detected toward mannobiose. In the initial stages of mannohexaose hydrolysis, a mixture of oligosaccharides was observed which is consistent with the proposed endo activity of the mannanase. The activity of MANA against carob galactomannan was pH-dependent and showed a maximum at pH approx. 7.0.

To determine the size of partially purified MANA derived from *E. coli* harbouring pKB6, the enzyme preparation was subjected to SDS/PAGE. A zymogram (using carob galactomannan as substrate) of the resultant 10% (w/v) polyacrylamide gel revealed a polypeptide of M_r 46000 with mannanase activity (Figure 3).

Recent studies have shown that some polysaccharidases exhibit remarkable resistance to proteolytic attack (Fontes et al., 1994). To evaluate whether MANA is resistant to proteolysis, a cell-free extract from *E. coli* harbouring pKB6 was incubated with pancreatin, a pancreatic extract containing the major proteinases produced by the exocrine pancreas, and retention of mannanase activity was monitored over 3 h. There was no decrease in MANA catalytic activity over the 3 h period, whereas three representative *E. coli* proteins, NADH oxidoreductase, malate

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-300
GGAAGGCCAGTGTTCCTGAGCCATATTCCTGGGTGTTTTCCTCCAGGAGCTAAGGG

CGGACGAATTGCGCTGTCTATTGATCTGGGATCCGTGTGCGCTACCAACCCCTAAGCGA

-200
AAGCAAGATGACGAATAATGCTAAGAAATGTAAGCAAAATAATCTCCAGGCACTGGCTG

TGTATAAAGCCGGTCTGGAATGCCAACCTGTGTGGCAACCTGATAACACAGCCGGG

-100
AATGACTGATTAACAAGATCGCCGAATCTTGATGTCTTGGGCGAATGCGCGATTGAGTG

+1
CAATCGCTGCCAGTAAGTCGTAATAAATCCGAGAGGACACAAATGAAACTATTACCA
H R T I T

CTGCAAGCCTTCATGGGCTGCGCAGAGCTTTCCTTGGGCTTTGCGCTGATAGCGCTGT
T A R L P W A A Q S F A L G I C L I A L

100
TGGGTTGTATACGCGCTAATAAATCATCGGCATCCAGGCGAGATGTCAAACCTGTA
L G C N H A A N K S S A S R A D V K P V

CGGTCAAATCGTGTATCCAGGCGACCATGAAACCCGTTCCCTGTTGCGTTTATGCT
T V K L L V D S Q A T M E T R S L F A P M

200
AGGAGCAGCGCAGACATTCATTATGTTTGGGCGACGAGAACACACCCGAGCTCA
Q E Q R R H S I M F G H Q H E T T Q G L

CTATTACCGCAGATGGTACCAATCCGATACCTTTAATGCGCTTGTGATTTTGCAG
T I T R T D G T Q S D T F N A V G D F A

300
CGGTCTATGTTGGGACAGCTGTCTATGTTGCCCTAAAGCGGAGGCGATATTGTG
A V Y G W D T L S I V A P K A G E D I V

400
CCCAAGTAAAAAGGCTTATGCGCGCGGGGCGATCATTACGTCGAGCTCGCATTTGTA
A Q V K K A Y A R G G I I T V S S H P D

ATCCAAAACCGATACCCAAAAGGCGTATGCGCCGTGGCCTCTCTGGGATCAAAACC
N P K T D T Q K G V W P V G T S W D Q T

500
CGGCTGTTGTGGATTCCCTGCGCTGGAGGCGCTATAACCCGCTATTAAATGGCTATCTG
P A V V D S L P G G A Y N P V L N G Y L

600
ACCAGGTGCGGAGTGGGCAATCACTGAAAGCAGCAGGCGCGCTGATACCGGTCA
D Q V A E W A N N L K D E Q G R L I P V

TCTTCGCTGTACCAAGAAATACCGGAGTGTGTTCTGGTGGGAGATAGCAATCAA
I F R L Y H E N T G S W F V D K Q S

700
CCCTGAACATACAAGCAATTGTTCCGCTATTCGTCGAGTATTGCGCGATGTGAAG
T P E Q Y K Q L F R Y S V E Y L R D V K

GTGTGCGCAATTCCTGTATGCTATTCGCCCAACAACTTTGGGAGCTGACCGAGGCG
G V R N F L Y A Y S P N N F W D V T E A

800
ATTACCTGGAGCGTTATCCGGGGGATGAGTGGTGGATGATGAGTATGATACCTATG
N Y L E R Y P G D E W V D V L G F D T Y

900
GCCCGGTGCGGACACGCTGACTGGTCCGCAATGGTGGCGCAATGCGCGCTTGGTTG
G P V A D N A D W F R N V V A N A A L V

CCGCTAGCGGAGAGCGCGGGGCAAAATACCGGTGATCTCCGAGATAGTATACGCGCTC
A R M A E A R G K I P V I S E I G I R A

1000
CGGACATTGAAGCGGGCTCTATGATAACCGATGATGCAAGCTTATCTCCGCGCTCA
P D I E A G L Y D N Q W I F Y R K L L T A R

AGGCCGATCCGATGACGCGAGATTGCTCTCTGCTGATGCGCAATGCCGCAAGG
K A D P D A R E I A F L L V W R N A R R

1100
AGTTCTGGGCGCAATGGCAGCGGTTCCCATTAATGGTGGCTGCTGCAACCGCGGA
E F L A P M A P R F I T G C L L T A R

1200
GAATATCAACAATGGCAGCTGGAGGACTTCAGGCGCTTTATGCGGATGAATTCACGC
R I S T H A P W R T S R P F M P M N S Q

GTTCAATCGGACATCGAGCAGGTCTATCAGCGTCCGACCTGATAGTTAAATAGTCTTT
R S I A T S S R S I S V R P

1300
TCTCTTGACTGATTCGCGCGCCAGCGGTGCGCGGGCGCGGAATTTGCTGCTTAACT

GATACCGGGATAACCGTAAAAATAGGTTAAATGTAGGACAATAATCTTATGGCGCTG

1400
TATTCTCCACGGTTTCAGGTTGATAAAAAATCCAATTCGCGCTATAGTGAGTCGTATT

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Figure 4 Nucleotide sequence of *manA*

The nucleotide sequence of *manA* and the deduced primary structure of MANA are shown. The positions of the two primers used to amplify *manA* are indicated by overlining the appropriate sequence. The experimentally determined N-terminal sequence of MANA is underlined.

dehydrogenase and β -galactosidase, had half-lives of less than 5 min under the same conditions. In the absence of pancreatin there was no decrease in the activity of MANA or the *E. coli*

B 27	-SEANGAA--LSNPANQTTKNVYSLANLPKNSKRVVSGHPGGYSDSTLAWIKQCARE
P 39	RADVFPVTVKLVDSQATMETRSLFAFMQE-QRRHSIMFGQH-ETTQGLTITRTDGTQSD
	*** * * * * *
B 84	LTGKMPGILSCDYKNWQTRLYVADQISYG-CNQLINFWNQGLVTISVHMPNPGFHSGE
P 97	TFNAV-GDFAAVY-GWDT-LSIVAPKAEGDIVAQVKKAYARGGIITVSSHFDNPKTDTQK
	* *
B 143	N-YKTILPSTSQFQNLTNHRTT-EGRRWKDMLDKMADGLDE---L-QNNG--VTVLFRPL
P 154	GVW-PV-GTS-WDQTPAVVDSLPGGAYNPVLNGYLDQVAEWANNLKDEQGRLLPVIFR-L
	* *
B 194	-HEMNGEWFVWGAEGYNQFDQTRANAYISAWR-DMYQYPTHERKLNLIWVYSPDVYRDH
P 210	YHENTGSWFWWG-D--KQ-S-T-PEQYKQLFRYSV-EYLKRVKGVNRLYAYSPNFWDV
	* *
B 252	V-TSY---YPGANYVDIALDSYHP--D-P---HSLTDQ--Y-NRMIAL-DK-PFAFAEI
P 263	TEANYLERYPGDEWVDVLGFDYGPVADNADWFRNVVANAALVARMARARGKIP-VISEI
	* *
B 297	G--PPESMAGSFD---YSNYIAIKQKYP--RTV-YFLAW-NDK--W-SPHNRR-GAWD-
P 322	GIRAPDIEAGLYDNQWYRKLISGLKAD-PDAREIAFLVWRNARREFLAPMAPRPTIIGC
	* *
B 343	LFND---S---WVVRNG--EIDYGQS-NPAT--V-L
P 381	LLTARRISTMAPWRTSRPFMPNHSQRSIATSSRSISV
	* *

Figure 5 Alignment of MANA with the mannanase from *Bacillus* sp.

The primary structures of MANA and the *Bacillus* mannanase are indicated by P and B, respectively. The position of the amino acid in the full-length sequence is indicated at the beginning of each line. Those residues that exhibit sequence identity or similarity are indicated by an asterisk.

enzymes over the 3 h period. These data clearly show that MANA exhibits unusual resistance to proteinases.

Nucleotide sequence of *manA*

The nucleotide sequence of the region of pKB1 containing *manA* was determined in both strands. Translation of the sequence, presented in Figure 4, revealed a single open reading frame of 1257 bp encoding a polypeptide of M_r 46938. The open reading frame had a G+C content of 54%, and a codon utilization similar to other *P. fluorescens* subsp. *cellulosa* genes encoding plant cell wall hydrolases (Ferreira et al., 1991, 1990). Upstream (6 bp) of the proposed translation initiation codon of *manA* was the sequence GAGGA, which is similar to prokaryotic ribosome-binding sequences (Hall and Gilbert, 1988). The deduced N-terminal sequence of MANA displayed many of the features typical of a bacterial signal peptide, with a basic hydrophilic eight-residue N-terminus followed by a stretch of 19 residues comprised primarily of small hydrophobic amino acids. The experimentally determined N-terminal sequence of recombinant MANA was XADVCKPVTVK, which is located directly downstream of Arg³⁹ (Figure 4). The deduced M_r of the mature enzyme (44000) is similar to the size of the recombinant mannanase detected in *E. coli* (Figure 3).

Comparison of the deduced primary structure of MANA with sequences in the SWISSPROT database showed that the enzyme exhibited greatest sequence identity (19%) with a mannanase from *Bacillus* sp. strain AM-001 (Akino et al., 1989; Figure 5). Using the classification of Henrissat (1991), MANA appears to belong to glycosyl hydrolase Family 26. Other members of this enzyme family include endoglucanases from *Bacillus subtilis* and *Bacteroides rumenicola*, and the catalytic domain of endoglucanase H from *Clostridium thermocellum*. One of the most highly conserved regions in all of the Family 26 enzymes is an aromatic amino acid-rich region with the consensus sequence WFWWG. Four residues downstream of this sequence are highly conserved histidine and glutamate residues.

Figure 6 SDS/PAGE of the purified MBP-MANA fusion protein

The fusion protein was purified by amylose affinity chromatography as described in the Materials and methods section. The samples, which were analysed by SDS/PAGE using a 10% (w/v) polyacrylamide gel, were as follows: purified *E. coli* MBP (lane 1); the purified fusion protein (lane 2); cell-free extract of *E. coli* containing pKB5 (lane 3). The gel was stained with Coomassie Blue and M_r values ($\times 10^{-3}$) of the fusion protein and MBP are shown.

Molecular architecture of MANA

All plant cell wall hydrolases from *P. fluorescens* subsp. *cellulosa* analysed to date have a modular structure, in which the individual domains are linked by sequences rich in hydroxy amino acids (Hazlewood and Gilbert, 1992). In contrast, MANA does not contain any obvious linker sequences or motifs characteristic of the non-catalytic domains of the *Pseudomonas* enzymes. These results indicate that the pseudomonad mannanase is comprised of a single catalytic domain, and lacks the non-catalytic CBDs located in other pseudomonad plant cell wall hydrolases. To evaluate whether this supposition is correct, the catalytic activity of the full-length mannanase was compared with the activity of truncated forms of MANA, containing small deletions from the C- and N-termini. Data presented in Figure 1 show that pKB6, which contains a PCR-amplified DNA sequence extending from -14 bp of the putative translational start codon of *manA* to 6 bp downstream of the translational stop codon of the gene, directed the synthesis of functional mannanase. Similarly, a derivative of the mannanase lacking 18 residues from the mature N-terminus (designated 18MANA) retained catalytic activity. Fusion of *manA'*, encoding 18MANA, to *malA* generated a protein of M_r 90000 which bound to amylose, could be eluted from the matrix with maltose and displayed mannanase activity (Figures 1 and 6). From the deduced sequence of MANA, the predicted M_r of the MBP-18MANA fusion protein is 85000. In contrast, derivatives of MANA lacking 54 (pKB4) and 16 residues (pKB7) from the N- and C-termini of the mannanase respectively did not exhibit catalytic activity (Figure 1). In addition, full-length MANA did not bind to cellulose (results not shown), suggesting that the enzyme does not contain a CBD. These data indicate that MANA is not a modular enzyme, but consists of a single catalytic domain.

DISCUSSION

Data presented in this paper provide the first description of mannan hydrolysis by *P. fluorescens* subsp. *cellulosa*, supporting the view that the bacterium is capable of degrading a wide range of plant structural polysaccharides. To investigate the enzymes involved in mannan hydrolysis, a gene encoding a *P. fluorescens*

subsp. *cellulosa* mannanase has been cloned, and the biochemical properties and molecular architecture of the enzyme have been characterized. The substrate specificity of MANA classified the enzyme as an endo- β -1,4-mannanase that displayed no detectable activity against other polysaccharides. This narrow substrate specificity is similar to the other plant cell wall hydrolases expressed by *P. fluorescens* subsp. *cellulosa*.

The molecular architecture of MANA is unusual in that it is the only *P. fluorescens* subsp. *cellulosa* plant cell wall hydrolase analysed to date not to contain a CBD. Ferreira et al. (1993) suggested that the CBD provides a mechanism by which a repertoire of plant cell wall hydrolases can assemble on the surface of the complex substrate, and through close association can increase the synergistic interactions between the different enzymes. The absence of a CBD in MANA suggests that its primary substrate is not a component of the plant cell wall.

Analysis of the primary structure of MANA placed the enzyme in glycosyl hydrolase Family 26 according to the classification of Henrissat (1991). Thus it appears that the pseudomonad mannanase, in common with the corresponding enzymes from *C. saccharolyticum*, *Bacillus* and *S. lividans*, has evolved from an ancestral sequence that gave rise to endoglucanases. It remains to be established whether MANA actually evolved from an endoglucanase, or whether the ancestral sequence was a β -glycosidase that independently gave rise to endoglucanases and mannanases. Interestingly, the three *P. fluorescens* endoglucanases (Hall and Gilbert, 1988; Gilbert et al., 1990; H. J. Gilbert, unpublished work) analysed to date do not belong to glycosyl hydrolase Family 26, suggesting that the *Pseudomonas* endoglucanases and MANA have not evolved from a common sequence. In contrast with mannanases, the xylanases characterized to date belong to two large enzyme families that almost exclusively consist of enzymes that attack only xylan. The only possible exocellulase in Family F does appear to exhibit substantially higher activity against xylan (Curry et al., 1988). It could be argued that, as mannose is structurally very similar to glucose compared with xylose, and further that some mannans have glucose moieties in the backbone, it is not surprising that mannanases and endoglucanases appear to be structurally similar. However, it should be noted that some endoglucanases exhibit substantial xylanase activity (Hall et al., 1988), and no cross-specificity between cellulases and mannanases has been reported [the endoglucanase and mannanase activities of *C. saccharolyticum* MANA are mediated by two distinct catalytic domains (Gibbs et al., 1992)]. In addition, hydrophobic cluster analysis suggests that the protein-folding of xylanases in Family G is similar to that of endoglucanases in Family H (Béguin and Aubert, 1994), a view supported by the conservation of the tertiary structure of two enzymes in Families G and H respectively (Okada, 1991). Thus despite the classification of endo-acting plant cell wall hydrolases into 11 families (Henrissat and Bairoch, 1993), some of the enzymes from different families do appear to be at least struc-

turally related. It is expected that analysis of the structure and function of mannanases in the future will verify whether the current hypothesis, that endo- β -1,4-mannanases have evolved from two ancestral sequences which also gave rise to endoglucanases, is correct.

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