β -Mannanase of *Streptomyces lividans* 66: cloning and DNA sequence of the *manA* gene and characterization of the enzyme

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The gene coding for a β -mannanase was cloned homologously from *Streptomyces lividans* and its DNA sequence was determined. The fully secreted enzyme was isolated and purified from culture filtrates of the hyperproducing clone *S. lividans* IAF36 grown in mineral salt media containing galactomannan as the main carbon source. It had a molecular mass of 36 kDa and a specific activity of 876 i.u./mg of protein. Under the assay

conditions used, the optimal enzyme activity was obtained at 58 °C and a pH of 6.8. The pI was 3.5. The kinetic constants of this mannanase determined with galactomannan as substrate were a $V_{\max}$ of 205 i.u./mg of enzyme and a K_m of 0.77 mg/ml. Data from SDS/PAGE and Western blotting show that the cloned enzyme was identical to that of the wild-type strain.

INTRODUCTION

Over the last few years, the use of xylanases for the biobleaching of wood pulps has attracted considerable interest. The replacement of the highly polluting chemical process used presently in the paper industry by enzyme technology would have a major impact on the environment. Besides xylanases, other hemicellulases such as β -mannanases might be of equal interest. This is especially the case for softwood pulps, in which mannans are the dominant hemicellulose fraction.

Analogous to xylan, mannan is a β -1,4-linked heteropolysaccharide consisting of a backbone of β -linked mannose residues which carries substitutions in the form of other sugars or of acids. Mannan is found abundantly in seeds of leguminous plants such as lucerne (*Medicago sativa*) and in the beans of carob trees (*Ceratonia siliqua*), in the form of galactomannan. In the softwood of gymnosperms, *O*-acetyl-galactoglucomannan is the major component of their hemicellulose fraction (Dekker, 1985).

Biodegradation of β -mannans is carried out by β -mannanases (1,4- β -D-mannan mannanohydrolase; EC 3.2.1.78), which attack the backbone of the polysaccharide, yielding short-chain oligomannosides. These sugars can then be hydrolysed further to mannose by the action of β -mannosidases (1,4- β -D-mannosidase; EC 3.2.1.25). Endo- β -mannanases have been purified and characterized from bacteria such as *Bacillus pumilus* (Akino et al., 1989), *Caldocellum saccharolyticum* (Lüthi et al., 1991) and *Streptomyces* spp. (Kusakabe and Takahashi, 1988). The enzyme has also been isolated from fungi such as *Polyporus versicolor* (Johnson and Ross, 1990), *Thielavia terrestris* (Araujo and Ward, 1990) and *Trichoderma reesei* (Ratto and Poutanen, 1988). The heterologous cloning of mannanase-encoding genes has been reported for a *Bacillus* sp. (Akino et al., 1989) and for *Caldocellum saccharolyticum* (Lüthi et al., 1991).

In our studies on the biobleaching of paper pulps by hemicellulases, we have cloned homologously several xylanase and cellulase genes of *Streptomyces lividans* (Mondou et al., 1986; Vats-Metha et al., 1990; Shareck et al., 1991; Théberge et al., 1992). The same gene bank was used to screen for genes coding

for other hemicellulases and for various debranching enzymes related to the xylanolytic system. In the present paper we describe the cloning and the DNA sequence of a mannanase gene (*manA*) as well as the overproduction, purification and characterization of the enzyme produced by clone *S. lividans* IAF36.

MATERIALS AND METHODS

Organisms

S. lividans 66 strain 1326 was obtained from Dr. D. A. Hopwood (John Innes Institute, Norwich, U.K.). The host strain used for cloning was a xylanase- and cellulase-negative mutant, *S. lividans* IAF10-164. The mutant was obtained by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, as described by Mondou et al. (1986). *S. lividans* 3131, containing the multi-copy plasmid pIJ702 (Katz et al., 1983) which served as cloning vector, was kindly supplied by Dr. E. Katz (Department of Microbiology, Georgetown University School of Medicine and Dentistry, Washington, DC, U.S.A.). *Escherichia coli* JM83 (*ara*, *lac-proAB*, *strA*, *thi-1*, Φ 80, *lacZ* M15) was used as host in all subcloning and DNA sequencing experiments. Phagemid pTZ18U was used as cloning vector and production of ssDNA was performed using the helper phage M13K07.

Cloning of the mannanase gene

The mannanase gene was isolated from a homologous gene bank previously constructed in the xylanase- and cellulase-negative mutant *S. lividans* 10-164 and containing approximately 25000 clones (Mondou et al., 1986). The screening was carried out on solid agar medium using 1% galactomannan as main carbon source. The mannanase-expressing clones were identified by the appearance of substrate clearing zones around the colonies.

DNA manipulations and sequencing

All DNA manipulations in *E. coli* and in *S. lividans* were carried out according to Maniatis et al. (1982) and Hopwood et al. (1985) respectively. Restriction fragments were subcloned into

Abbreviations used: LBG, locust bean gum; DNS, dinitrosalicylic acid.

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The nucleotide sequence data reported in this paper will appear in the GenBank, EMBL and DDBJ Nucleotide Sequence Databases under accession no. M92297.

phagemid pTZ18U and appropriate deletions of the 5'-end of the insert carrying the gene were performed using the Double-stranded Nested Deletion Kit from Pharmacia-LKB. Single-stranded DNA was prepared from overlapping deleted phagemids and sequenced by the chain-termination dideoxy method of Sanger et al. (1977). To fill the remaining gaps, oligonucleotide primers were synthesized using a Gene Assembler (Pharmacia-LKB). The 5'-end sequence was extended by plasmid sequencing using an oligonucleotide primer. DNA sequences were assembled and analysed using the Pustell DNA analysis programs from International Biotechnology Inc., and DNASIS and PROSIS software from Pharmacia-LKB.

Culture conditions

The strains and mannanase-positive clones resulting from the transformations were maintained on yeast extract/malt extract/maltose (YEME) agar slants containing 50 µg/ml thiostrepton (obtained as a gift from Squibb Canada Ltd., St. Laurent, Québec, Canada). Spore suspensions prepared from such cultures in 10 ml of 0.9% NaCl were used to inoculate 100 ml of trypticase soy broth in a 500 ml Erlenmeyer flask containing 5 µg/ml thiostrepton. Incubation was carried out at 34 °C on a rotary shaker at 240 rev./min for about 30 h, and 6% of this pre-culture served to inoculate the fermentation medium. The basic composition of the latter has been described previously (Bertrand et al., 1989). Galactomannan from locust bean gum (LBG) (Sigma Chemical Co., St. Louis, MO, U.S.A.) with a galactose/mannose ratio of 1:4 was used as the main carbon source at 1% (w/v). Incubations were carried out at 34 °C in 500 ml Erlenmeyer flasks containing 100 ml of culture medium on a rotary shaker rotating at 240 rev./min for 48–72 h. For large-scale enzyme production, 2-litre Erlenmeyer flasks containing 400 ml portions of the medium were used.

Enzyme recovery

The fermentation broths were filtered through diatomaceous earth (filter aid) in a Buchner funnel and the mycelium was discarded. Crude enzyme preparations were obtained by precipitation with 3 vol. of cold 95% ethanol. The precipitate, allowed to settle overnight at 4 °C, was recovered by filtration through a porous glass Buchner funnel. This crude enzyme preparation was either used directly for the purification or stored after drying with acetone. In the dried form it did not lose activity when stored at 4 °C for several months.

Enzyme purification

For purification of the mannanase, the precipitate (27 g) obtained from 8 litres of culture filtrate was dissolved in 40 ml of 20 mM Tris/HCl buffer, pH 8.5, and dialysed overnight against the same buffer. The solution was centrifuged at 16000 *g* to remove any undissolved particles, diluted with 40 ml of the buffer and passed through a 0.22 µm Acrodisc filter (Gelman Sci., Ann Arbor, MI, U.S.A.). The filtrate was adsorbed directly on to a semi-preparative anion-exchange h.p.l.c. column (DEAE-5-PW; Waters-Millipore, St. Laurent, Québec, Canada) which had been equilibrated with the above-mentioned Tris/HCl buffer. The enzyme was eluted in the same buffer with a linear gradient of 0–1 M NaCl. The elution of the proteins was monitored at 280 nm. The mannanase activity contained in the fractions was determined by the cup-plate diffusion assay on mineral salt agar

media (Daigneault-Sylvestre and Kluepfel, 1979) containing 1% (w/v) galactomannan. The visibility of the substrate-clearing zones could be improved by Congo Red staining (Teather and Wood, 1982). The active fractions were pooled and dialysed against distilled water at 4 °C. Pure mannanase was obtained after lyophilization of this solution.

Preparation of the enzyme substrate

LBG was used as substrate in the enzyme assays. It was purified in the following manner: 10 g of LBG was suspended in 200 ml of 80% (v/v) ethanol and heated for 10 min in a boiling-water bath. The undissolved material was recovered by filtration and thoroughly rinsed with additional ethanol, resuspended in 200 ml of cold distilled water, left overnight, and then an additional 300 ml of water was added. The mixture was agitated vigorously in a Waring blender for 2 min and centrifuged for 30 min at 3000 *g*. The water-soluble galactomannan contained in the supernatant was precipitated by addition of 2 vol. of ethanol and dried with acetone.

Enzyme assays

The 1,4- β -D-mannanase activity was determined by adapting the dinitrosalicylic acid (DNS) method described for xylanases (Kluepfel et al., 1991). Galactomannan from LBG served as substrate.

Enzyme assays were carried out by mixing 1 ml of appropriately diluted samples with 1 ml of 0.1 M McIlvaine buffer, pH 6.7, containing 0.5% LBG. The mixture was incubated at 58 °C for 10 min. The reducing sugars released were then determined by the DNS method against a standard curve obtained with mannose.

α -Galactosidase activity was tested with *p*-nitrophenyl α -D-galactopyranoside (Sigma) as substrate, using the above conditions. Liberation of *p*-nitrophenol was determined spectrophotometrically at 400 nm.

Enzyme activities were expressed in i.u., where 1 unit is the amount of enzyme that releases 1 µmol of reducing sugars (expressed as mannose) in 1 min. All results reported are the averages of at least two independent experiments.

Protein

The protein content of enzyme preparations was determined according to Lowry et al. (1951) using BSA fraction V (Sigma) as standard.

Determination of isoelectric point

Analytical isoelectric focusing was carried out on PhastGel containing Pharmalyte carrier ampholytes in the pH range 3–10 using the Phastsystem (Pharmacia). The gels were silver-stained after the run.

The electrophoretic titration method of Pharmacia-LKB Biotechnology (Pharmacia-LKB Separation and Development Technique Files No. 100 and 210) and silver staining were used to check the purity of mannanase.

Determination of molecular mass

The molecular mass of the purified mannanase was estimated by SDS/PAGE as described by Laemmli (1970). The protein bands were detected by silver staining. The molecular mass was confirmed by h.p.l.c. using two PW 300 ProteinPak columns

Figure 1 Restriction maps of plasmids pIAF3, pIAF36 and pIAF61

The open boxes represent the inserts cloned into plasmid pIJ702 (thin line). The closed box in pIAF3 represents the 1.7 kb *SphI* fragment which served as a probe for hybridization and DNA sequencing. The arrow marked A indicates the orientation of the *manA* structural gene. The broken-line arrows correspond to the positions of the gene in restriction fragments which hybridize with the probe. The open arrows show the transcription of the *melC* gene contained in plasmid pIJ702. Restriction endonucleases: Ba, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; Sa, *Sau*3AI; Sp, *Sph*I; Ss, *Sst*I.

(Waters) in series, calibrated with standard proteins (Combithek II; Boehringer Mannheim).

Hydrolysis of manno-oligomannosides

The mannohexaose (M_6) that served as substrate for establishing hydrolysis patterns, as well as the smaller manno-oligosides mannobiose (M_2) to mannopentaose (M_5), serving as standards, were purchased from Megazyme Ltd. (Sydney, Australia). The enzymic hydrolysis of this substrate was carried out in a volume of 500 μ l containing 1 mg/ml substrate dissolved in 0.1 M McIlvaine buffer (pH 6.7) and 8.7 i.u. (corresponding to 10 μ g) of the purified enzyme. After incubation periods lasting from 7 min to 24 h at 58 $^{\circ}$ C, the enzyme was inactivated in a boiling-water bath for 10 min. The sample was centrifuged and filtered through a 0.45 μ m-pore-size Acrodisc. Portions of 10 μ l of this filtrate were analysed by h.p.l.c. on an Aminex HPX-42A column (Kluepfel et al., 1991).

Antibodies and Western blotting

Antibodies were raised against mannanase in New Zealand White rabbits by injecting 50 μ g of pure enzyme in complete Freund's adjuvant. Three additional injections of 100 μ g of mannanase, in incomplete adjuvant, were administered at intervals of 1, 2 and 7 weeks respectively.

Western blotting was carried out using anti-mannanase antibodies coupled to ^{125} I-Protein A (Amersham Canada Ltd., Oakville, Ontario, Canada) as previously reported (Mondou et al., 1986).

RESULTS

Cloning and localization of the mannanase gene

Screening of a gene bank of *S. lividans*, constructed in *S. lividans* IAF10-164 as described by Mondou et al. (1986), resulted in the isolation of three clones showing mannanase activity on LBG plates. These clones harboured plasmids pIAF3, pIAF36 and pIAF61, which had chromosomal DNA insertions of 4.3 kb, 10 kb and 4.1 kb respectively. Restriction mapping analysis of

Table 1 Comparison of β -mannanase production by wild-type and host strains of *S. lividans* 66 and the clones carrying *manA* recombinant plasmids pIAF3, pIAF36 and pIAF61 after cultivation in submerged cultures for 72 h

<i>S. lividans</i> strain or clone	Activity (i.u./ml)
1326 (wild type)	1
3131 (wild type + pIJ702)	1
10-164 (<i>xln</i> ⁻ , <i>cel</i> ⁻)	1
IAF3	2
IAF36	63
IAF61	6

the inserts of the recombinant plasmids and Southern hybridization revealed identical sequences in all three clones (Figure 1). β -Mannanase activity was determined for each of them and compared with that of the wild-type and the mutant serving as host strain (Table 1). All were mannanase-positive, but the three selected clones showed improved enzyme production. Of these, *S. lividans* IAF36 produced mannanase at a level 60 times that of the wild type, and thus was chosen for further enzyme purification. A small 1.7 kb *SphI* fragment from pIAF3, which was found in all three clones, served for the determination of the DNA sequence of the *manA* gene.

Nucleotide sequence analysis

The nucleotide sequence of the 1.7 kb *SphI* fragment from pIAF3 and the immediate 5'-end was determined. The sequence from nt 1705 to the end is the sequence of the tyrosinase gene of the cloning vector. This is not surprising, since the genomic bank of *S. lividans* 1326 was constructed by partially digesting the chromosomal DNA with restriction endonuclease *Sau*3A and cloning these fragments into pIJ702 digested with *Bgl*II (Mondou et al., 1986). The analysis of the remaining DNA sequence revealed a large open reading frame starting from an ATG codon located at nt 214 to a TGA termination codon at position 1303 (Figure 2). This open reading frame is preceded by a putative

nt																	aa
1	GGC	GCC	CCC	TCT	TTT	TAC	TAA	ACC	CGT	AAA	CGC	GCT	GAC	CAG	AGT	GTG	
49	AAG	CCC	GCG	TAT	CCA	GGT	CGT	TCA	CCT	GCG	TCA	ACT	GCC	CCT	GCA	TGA	
97	CGG	ATT	GAC	AGG	CCT	GCC	AGG	TGT	CCC	TAC	GGT	CCC	TAT	CGG	TTA	ACC	
145	GGT	AAA	TGT	CGC	CCC	GAG	GTG	CGG	GAT	AAC	GTT	GTC	AGG	<u>TGC</u>	<u>ATG</u>	<u>CTC</u>	
193	GCC	CGC	CAT	CGA	AGG	AGA	CAC	ATG	CGT	AAC	GCA	AGA	AGC	ACG	CTG	ATC	
								M	R	N	A	R	S	T	L	I	9
241	ACG	ACC	GCG	GGG	ATG	GCG	TTC	GCC	GTT	CTG	GGC	CTG	CTG	TTC	GCA	CTC	
	T	T	A	G	M	A	F	A	V	L	G	L	L	F	A	L	25
289	GCC	GGT	CCT	TCC	GCC	GGA	AGG	GCG	GAG	GCC	GCG	GCC	GGT	GGC	ATC	CAC	
	A	G	P	S	A	G	R	A	E	A	<u>T</u>	<u>A</u>	<u>A</u>	<u>G</u>	<u>G</u>	<u>I</u>	41
337	GTC	AGC	AAC	GGA	CGT	GTC	GTC	GAG	GCG	AAC	GGC	AGC	GCG	TTC	GTC	ATG	
	<u>V</u>	<u>S</u>	<u>N</u>	<u>G</u>	<u>R</u>	<u>V</u>	<u>V</u>	<u>E</u>	<u>G</u>	<u>N</u>	<u>G</u>	<u>S</u>	<u>A</u>	<u>F</u>	<u>V</u>	<u>M</u>	57
385	CGC	GGG	GTC	AAC	CAC	GCC	TAC	ACC	TGG	TAT	CCC	GAC	CGC	ACC	GGC	TCC	
	R	G	V	N	H	A	Y	T	W	Y	P	D	R	T	G	S	73
433	ATC	GCC	GAC	ATC	GCG	GCC	AAG	GGA	GCC	AAC	ACC	GTC	CGC	GTC	GTG	CTC	
	I	A	D	I	A	A	K	G	A	N	T	V	R	V	V	L	89
481	AGC	AGC	GGC	GGC	CGC	TGG	ACG	AAG	ACG	AGC	GCG	TCC	GAG	GTC	TCG	GCG	
	S	S	G	G	R	W	T	K	T	S	A	S	E	V	S	A	105
529	CTC	ATC	GGC	CAG	TGC	AAG	GCC	AAC	AAG	GTC	ATC	TGT	GTC	CTT	GAG	GTG	
	L	I	G	Q	C	K	A	N	K	V	I	C	V	L	E	V	121
577	CAC	GAC	ACC	ACC	GGC	TAC	GGC	AAG	GAC	GGC	GCG	ACC	AGC	CTG	GAC	CAG	
	H	D	T	T	G	Y	G	K	D	G	A	T	S	L	D	Q	137
625	GCC	GGC	GAC	TAC	TGG	GTC	GGT	GTG	AAG	AGC	GCC	GCC	TGG	AGG	GCC	CAG	
	A	G	D	Y	W	V	G	V	K	S	A	A	W	R	A	Q	153
673	GAG	GAC	TAC	GTC	GTC	GTC	AAC	ATC	GGC	AAC	GAA	CCC	TTC	GGC	AAC	ACC	
	E	D	Y	V	V	V	N	I	G	N	E	P	F	G	N	T	169
721	AAC	TAC	GCC	GCG	TGG	ACC	GAC	GCG	ACC	AAG	TCG	GCC	ATC	GGC	AAG	CTC	
	N	Y	A	A	W	T	D	A	T	K	S	A	I	G	K	L	185
769	CGC	GGC	GCC	GGT	CTC	GGC	CAC	GCC	CTG	ATG	GTC	GAC	GCG	CCC	AAC	TGG	
	R	G	A	G	L	G	H	A	L	M	V	D	A	P	N	W	201
817	GGC	CAG	GAC	TGG	TCC	GGC	ACG	ATG	CGG	TCC	AAC	GCG	GCC	TCG	GTC	TTC	
	G	Q	D	W	S	G	T	M	R	S	N	A	A	S	V	F	217
865	GCC	TCC	GAC	CCC	GAC	CGC	AAC	ACC	GTC	TTC	TCC	ATC	CAC	ATG	TAC	GGG	
	A	S	D	P	D	R	N	T	V	F	S	I	H	M	Y	G	233
913	GTC	TAC	GAC	ACC	GCC	GCC	GAG	GTG	CGG	GAC	TAC	CTG	AAC	GCC	TTC	GTC	
	V	Y	D	T	A	A	E	V	R	D	Y	L	N	A	F	V	249
961	GGC	AAC	GGA	CTG	CCC	ATC	GTC	GTC	GGC	GAG	TTC	GGC	GAC	CAG	CAC	AGT	
	G	N	G	L	P	I	V	V	G	E	F	G	D	Q	H	S	265
1009	GAC	GGC	AAC	CCC	GAC	GAG	GAC	GCC	ATC	ATG	GCG	ACG	GCC	CAG	TCC	CTC	
	D	G	N	P	D	E	D	A	I	M	A	T	A	Q	S	L	281
1057	GGC	GTC	GGC	TAC	CTC	GGC	TGG	TCC	TGG	AGC	GGC	AAC	GGC	GGC	GGC	GTC	
	G	V	G	Y	L	G	W	S	W	S	G	N	G	G	G	V	297
1105	GAG	TAC	CTC	GAC	ATG	GTC	AAC	GGC	TTC	GAC	CCG	AAC	TCG	CTG	ACG	AGC	
	E	Y	L	D	M	V	N	G	F	D	P	N	S	L	T	S	313
1153	TGG	GGC	AAC	CGC	ATC	CTC	TAC	GGC	AGC	AAC	GGC	ATC	GCC	GCG	ACG	TCC	
	W	G	N	R	I	L	Y	G	S	N	G	I	A	A	T	S	329
1201	AGG	ACG	GCC	ACC	GTG	TAC	GGC	GGT	GGT	GGC	GGC	AGC	ACG	GGC	GGC	ACC	
	R	T	A	T	V	Y	G	G	G	G	G	S	T	G	G	T	345
1249	GCG	CCC	AAC	GGT	TAC	CCG	TAC	TGC	GTG	AAC	GGC	GGT	GCC	GTC	CGA	CCC	
	A	P	N	G	Y	P	Y	C	V	N	G	G	A	V	R	P	361
1297	GGA	CGG	TGA	CGG	GTG	GGG	CTG	GGA	GAA	CAG	CCG	GTC	CTG	CGT	CGT	CCG	
	G	R	*														
1345	GGG	CAG	CGC	GGC	GGA	CCA	CTG	ACC	CGA	CCG	ACG	GCG	ACG	CCC	CAC	CCT	
1393	GGG	GTT	CCG	GGG	TCC	GGG	GCC	GTT	TCC	AGT	GAT	TCG	GGC	CCA	CGC	CGT	
1441	GGG	GAG	ACG	GCG	AGC	TGG	GCG	TGT	GCG	GCG	CCT	TCG	AGT	CGC	GGA	TGT	
1489	GGA	TCG	CGT	CCG	GGC	GGG	CCG	CCA	CCT	CGA	CAC	AGT	CGC	CGT	CAC	CGC	
1537	TGC	TGC	TGT	AGC	TCG	ACT	TGT	GCC	AGG	ACA	GGG	CGA	TTT	CCA	CGC	AGT	
1585	CGC	CGT	CGC	CAC	CGC	TGC	TGT	ACG	AGC	TTT	TGA	ACC	AGG	CCA	GTT	CCG	
1633	TCG	TGT	TCA	TGA	GGC	TCC	TCG	CAT	CCG	CCG	CAA	ACG	GCC	CAG	GGA	GTC	
1681	GTC	GAG	GGA	GAG	GGG	CCT	<u>GTG</u>	<u>ATC</u>	TCG	TCG	AAG	GCG	GCG	GGG	GCG	CCG	
1729	GAC	GCG	GCC	GGG	TTC	CCG	GGG	ACC	TCG	GGG	GTG	TGG	TGC	CCC	CGA	TCG	
1777	TCC	GCG	CGG	GCG	CCG	GGA	AGG	GCG	ACC	AGG	GGG	ACA	CCG	GCG	GCG	ACG	
1825	ACG	GCT	GCG	GCG	CCG	AGC	GCG	CGA	CGA	CGG	GTG	AGT	TCC	GGC	<u>ATG</u>	<u>C</u>	

Figure 2 Nucleotide sequence and deduced amino acid sequence of *manA*

The amino acids that are identical to the N-terminus of the mature protein are underlined (residues 36–42). The small arrow indicates the cleavage site of the signal peptidase. The putative ribosome-binding site is shown in bold face. The restriction sites *Sph*I and *Sau*3A are underlined.

ribosome-binding site (5' AGGAG 3') located 9 nt upstream from the translation codon which shows perfect complementarity to the 3'-end of the 16 S rRNA of *S. lividans* (Bibb et al., 1984). The polypeptide encoded by this 1089 nt open reading frame contains 363 amino acids, giving a molecular mass 37602 Da. The base composition of the coding region of *manA* (68.8% G+C) is in good agreement with the G+C content of *Streptomyces* DNA, with more than 92% C or G at the third position. Accordingly, analysis of codon usage reveals that 18 codons out of 61 are not used (results not shown). The first five amino acids of the N-terminus of the purified mature extracellular mannanase were determined (underlined amino acid residues in Figure 2) using the Edman method (carried out by Dr. A. Bell at the Sheldon Biotechnology Centre of McGill University, Montréal, Canada). This allowed us to establish the reading frame of the DNA sequence and enabled us to deduce that the precursor protein contains a signal peptide of 35 amino acids and molecular mass 3489 Da. As previously reported for extracellular proteins of Gram-positive bacteria, the signal peptide is quite long (Shareck et al., 1991). Thus, after processing, the mature mannanase shows a molecular mass of 34113 Da, which matches closely the one estimated by SDS/PAGE (see Figure 4). The signal peptide of the mannanase pre-protein shows an amino acid composition and features normally found in such sequences (Perlman and Halvorson, 1983), i.e. a positively charged hydrophilic N-terminus containing arginine residues, followed by a long hydrophobic domain, and a helix-breaking proline residue located at position -7, allowing a turn to the polypeptide which presumably exposes the cleavage site Ala-Glu-Ala to the signal peptidase.

Amino acid sequence identity

The deduced amino acid sequence of the *manA* protein of *S. lividans* was compared with the two reported to date from other sources. As shown in Figure 3, the streptomycete mannanase exhibited 69% amino acid similarity to that of the mannanase from *Caldocellum saccharolyticum* (Lüthi et al., 1991), but no significant similarity was found to the β -mannanase isolated from *Bacillus* (Akino et al., 1989).

Enzyme production

The β -mannanase was produced in submerged cultures with clone *Streptomyces lividans* IAF36 using the mineral salt medium M13 containing 1% (w/v) galactomannan as the main carbon source. Optimal enzyme levels of 87 i.u./ml were reached after 96 h of incubation. The addition of 0.2% (v/v) commercial olive oil increased significantly the mannanase secretion (by more than 30%). A similar effect had been shown previously by Bertrand et al. (1989) for the production of xylanase A by *S. lividans*. All of the enzyme activity was secreted into the culture filtrates. Cell extracts prepared by sonication of the mycelium contained only trace amounts of activity, possibly reflecting some transient mannanase before secretion.

Enzyme purification

Crude enzyme preparations in the form of acetone-dried powder contained a 4-fold increase over mannanase activity in the culture filtrate. Further purification was achieved by h.p.l.c. on a DEAE-cellulose column. The purified β -mannanase was eluted

	1	10	20	30	40	50	60
ManA S.L.	MRHARSTL	---ITTA	GHAPAVLGLL	PALAGPSA	GR	AREAAAG	---GTHVSHGRVVRGM
ManA C.S.	MRL	RTKIRKWL	SLVCTVVF	LLNLF	TAHVTLFVVG	AATSDGV	V---KI-DT
	61	70	80	90	100	110	120
ManA S.L.	GSAPVNRGVN	HAYTNY	PDRT	---GSI	ADIAAG	A	---NTVRVVLSSGGRTKTSASEVSALI
ManA C.S.	ST	---LIGT	---MHACWTRDR	LDTALNG	---	RSWGHNSVRVLS	MGYRWTKIPASEVANII
	121	130	140	150	160	170	180
ManA S.L.	QCKAN	---EVICVLE	VEDTTGYG	KDGAT	---SLDQAGD	TVNGVKS	AAWRAQEDYVVVNIGNE
ManA C.S.	SLSRSLGPKAI	---	ILEVHDTTGYG	EDGAACSLAQ	AVETWKEIKSVLD	---	GHEDFVIINIGNE
	181	190	200	210	220	230	240
ManA S.L.	PFCHNTYA	AMTDATK	SAIGK	---	LRGAGLGH	ALNVDP	HHNGQWSSGTHKSHAAVSFASDPDR
ManA C.S.	PYGNHNY	QHWVNDTKAI	---	KALRDAG	FKHTIMVD	APHNGQWSSGTHKSHAAVSFASDPDR	---
	241	250	260	270	280	290	300
ManA S.L.	NTVFSI	HNHYGVYD	TAELVRYL	NAFVGR	---	GLPIVVG	EPDQSDGNFORDAIMATA
ManA C.S.	MLVFSI	HNHYGVYNT	ASKVSEYIK	SFV	---	DKGLPLVIG	EPDQSDGNFORDAIMATA
	301	310	320	330	340	350	360
ManA S.L.	LGVGYL	GWNSG	MOGGVET	LDHVMG	PDH	---	SLTSWNGHLYSGMIAATSTATVYGOOG
ManA C.S.	IGL	---	F	---	SWWONGSSY	VGYL	LDHVMGPDHNP
	361	370	380				
ManA S.L.	OSTOGT	APMGYPYC	VNOGAVR	PGR			
ManA C.S.	VTPTPP	PPR	---	Q	---	H	---

Figure 3 Optimal alignment of the amino acid sequences of the mannanases of *S. lividans* 1326 and *C. saccharolyticum*

Identical amino acids are marked by two dots (:), while a single dot indicates conservative changes. ManA S.L., β -mannanase from *S. lividans*; ManA C.S., β -mannanase from *C. saccharolyticum*.

Figure 4 SDS/PAGE of purified β -mannanase from *S. lividans* IAF36

Lane 2 was loaded with 5 μ g of β -mannanase from *S. lividans* IAF36, and the gel was silver-stained. Standard protein markers were run in the lane 1: phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa).

from the column with a linear gradient of 0–1 M NaCl at a salt concentration of 21%. This two-step method led to a 10-fold purification of the enzyme at a yield of 29%. The purity of the mannanase was verified by SDS/PAGE followed by silver staining (Figure 4) and by the electrophoretic titration method (Pharmacia; results not shown). Pure β -mannanase was recovered as a white powder, after dialysis against distilled water and lyophilization. The enzyme can be stored at 4 °C without loss of activity.

Characterization of the enzyme

The cloned β -mannanase from *S. lividans* IAF36 was compared with that of *S. lividans* 1326, 3131 and IAF10-164. SDS/PAGE and Western blotting with anti-mannanase antibodies showed that the purified enzyme was identical to that of the wild-type and mutant strains (Figure 5). The apparent molecular mass of

Figure 5 Western blot analysis of culture filtrates (100 µl) using anti-mannanase antibodies

Lanes: A, *S. lividans* 1326 (wild type); B, *S. lividans* 3131 (wild-type containing pIJ702); C, *S. lividans* IAF10-164 (*cel⁻*, *xln⁻* mutant); D, *S. lividans* IAF36 (*manA*); E, pure β -mannanase (10 µg); st., ^{14} C-labelled protein standards.

the β -mannanase determined by SDS/PAGE was estimated to be 36 kDa by comparison with a standard curve drawn from the migration of proteins with known molecular masses. The monomeric nature of the enzyme was confirmed by h.p.l.c. on a molecular sieve calibrated with appropriate protein standards.

Analytical isoelectric focusing of the enzyme on a pH gradient of 3–10 indicated a pI of 3.5. The mannanase was active between 35 and 70 °C with an optimum at 58 °C, and within a pH range of 5.5–8.0 with a maximum at pH 6.7. Enzyme incubated in absence of substrate was stable at temperatures between 0 and 38 °C. At 42 °C the half-life was about 4 h, while at 58 °C the mannanase lost its activity within 15 min. In the presence of 1% galactomannan at 60 °C, enzyme hydrolysis proceeded linearly for more than 30 min, indicating increased stability of the β -mannanase in the presence of its substrate.

The kinetic constants of the purified β -mannanase were determined under the assay conditions of pH 6.7 and 58 °C and

using either the Lineweaver–Burk or the Eadie–Hofstee methods. The substrate range was 0.5–5.0 mg/ml. The averages of four independent determinations were: V_{max} , 207 i.u./mg of enzyme and K_m 0.77 mg/ml. The enzyme did not show any xylanase, endocellulase or α -galactosidase activity.

Hydrolysis studies were carried out on mannohexaose at a concentration of 1% under optimal assay conditions using 8.5 i.u. of the pure β -mannanase from *S. lividans* IAF36. As shown in Figure 6, after 7 min the hexasaccharide has been rapidly hydrolysed into mannobiose, mannotriose and mannotetraose. Further incubation of up to 24 h transformed these oligomers into mainly mannobiose, with small amounts of mannose and mannotriose as end products.

DISCUSSION

The β -mannanase gene of *Streptomyces lividans* 66 was cloned in the cellulase- and xylanase-negative but mannanase-positive mutant *S. lividans* IAF10-164 using the multi-copy plasmid pIJ702. A significant overexpression of the *manA* gene was observed in clone IAF36, amounting to more than 60 times that of the wild-type strain. This was similar to the previously reported expressions for the genes of xylanases A, B and C (Mondou et al., 1986; Vats-Mehta et al., 1990; Kluepfel et al., 1991) and for that of an endoglucanase (Thberge et al., 1992). The other mannanase-positive clones did not show such a high production (Table 1). In the case of *S. lividans* IAF3 this could be explained by the proximity of the *manA* gene to the *melC* gene (coding for melanin synthesis) contained in the vector pIJ702 which is transcribed in a converging orientation. This configuration might lead to the synthesis of overlapping transcripts (anti-sense mRNA) competing with the sense mRNA encoding the mannanase (Chater and Hopwood, 1989). This is not the case for pIAF36, in which the *manA* gene was mapped at the opposite end of a large 10 kb insert (Figure 1). In pIAF61, where *manA* is read in the same direction as *melC*, the gene might not have been cloned in its entirety. This plasmid has not been investigated further. Cloning of genes coding for mannanases have been reported for a *Bacillus* sp. (Akino et al., 1989), and more recently

Figure 6 Hydrolysis of mannohexaose (M_6) by purified β -mannanase from *S. lividans* IAF36 over a period of 24 h

Mannohexaose (1 mg/ml), dissolved in 0.1 M McIlvaine buffer, pH 6.7, was hydrolysed with 10 µg of purified β -mannanase. Samples of 10 µl were withdrawn at intervals of 7, 180 and 1440 min (24 h) and analysed by h.p.l.c. as described in the Materials and methods section. M_1 , mannose; M_2 , mannobiose, etc.

for *Caldocellum saccharolyticum* (Lüthi et al., 1991). In both cases a heterologous approach had been employed with *E. coli* as host. The expression of the mannanase from *C. saccharolyticum* was reported to be 0.17–6.8 i.u./mg of protein.

S. lividans IAF36 produced its mannanase on galactomannan from LBG, causing rapid liquification of this viscous substrate. The complete hydrolysis of this carbohydrate also requires the presence of an α -galactosidase, which is produced by both the wild-type strain and the clone. Since the mannanase levels produced by the wild-type strain were fairly low, the enzyme was purified from the culture filtrates of *S. lividans* IAF36 carrying multi-copies of the *manA* gene. The identity of this protein with that of the wild-type was verified by Western blotting using anti-mannanase antibodies which had been prepared in rabbits using the purified recombinant enzyme (Figure 5). This is an indication that the overproducing clone carries on plasmid pIAF36 the complete gene and is expressing the wild-type enzyme. This was further substantiated by analysis of the amino acid sequence deduced from the *manA* gene, which showed significant similarity with that of the mannanase identified by Lüthi et al. (1991). Since *S. lividans* IAF36 does not produce any endoglucanase activity and the cloned mannanase is fully secreted into the culture filtrates, this clone is of considerable industrial potential for the biobleaching of softwood pulp in paper manufacturing, as described by Viikari et al. (1986). Significant improvements in mannanase yields should be expected once the physiological parameters for optimal gene expression have been studied in a manner similar to that for xylanase A (Bertrand et al., 1989).

The biochemical characteristics of the *S. lividans* mannanase are similar to those reported for many of the enzymes of both eukaryotic and prokaryotic origins. They are, however, difficult to compare since various substrates of different origin were used for the determination of these constants. Not surprisingly, a close similarity was observed with enzymes of *Streptomyces* sp. such as mannanase IV described by Takahashi et al. (1984). The thermostability of the enzyme is in the mesophylic range, but improved significantly in the presence of a substrate such as LBG, permitting prolonged hydrolysis at temperatures as high as 60 °C. From the preliminary hydrolysis studies carried out with linear unsubstituted mannohexaose, a typical endo-enzyme activity is indicated, resulting in the rapid formation of mainly mannobiose as the end product (Figure 6). After 7 min of incubation the appearance of a mannopentaose peak and possibly of small amounts of mannoheptaose (shoulder), in the absence of mannose, suggests the possibility of a transglycosylation reaction at this stage of hydrolysis.

The deduced amino acid composition of the β -mannanase A of *S. lividans* revealed that this protein is very hydrophilic, having 8.8% acidic, 8.5% basic and more than 44% polar amino acids. Moreover, the protein contains 15.8% glycine residues. Comparison of the amino acid sequence of the mannanase of *S. lividans* with the two reported sequences revealed 68.8% identity with that of *C. saccharolyticum*. The latter, however, is slightly less hydrophilic (38%) and contains only 7% glycine residues. This amino acid similarity suggests that these enzymes are evolutionarily conserved and that no deleterious amino acid changes were allowed. However, the proline/

threonine-rich region (PT-box), described at the C-terminus of the *C. saccharolyticum* mannanase and assumed to be involved in recombination events in this micro-organism, is not found in the *S. lividans* mannanase. The PT-box is replaced by a stretch of glycine residues. The significant amino acid identity between the two mannanases is quite surprising, since the two micro-organisms have very different G+C contents. The mannanase gene of *C. saccharolyticum* has a G+C content of only 43% (Lüthi et al., 1991), compared with 69% for the *manA* gene of *S. lividans*. This observation is an example of the flexibility of the genetic code, especially at the wobble position, as reviewed recently by Osawa et al. (1992).

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