Purification of the acidic pectate lyase and nucleotide sequence of the corresponding gene (*pelA*) of *Erwinia chrysanthemi* strain 3937

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The *pelA* gene from *Erwinia chrysanthemi* strain 3937, which encodes the acidic pectate lyase, PLa, has been sequenced and characterized. The structural gene consists of a 1179 bp open reading frame encoding a polypeptide of 41555 Da, which includes an N-terminal signal peptide. The deduced amino acid sequence shows a protein very similar to some PLs already sequenced. Cloning of the *pelA* gene behind the *lacZ* promoter of the vector pTZ19R allowed overexpression of PLa into a derivative of strain 3937 deleted of the other *pel* genes. The mature protein was obtained in milligram amounts from the supernatant of this strain and at homogeneous purity after two purification steps. Its biochemical properties were similar to those of other PLs. Polyclonal antibodies raised against the purified PLa cross-reacted with the basic pectate lyase PLd, but not with PLe. The role of PLa in pathogenicity is discussed.

Introduction

The enterobacterium *Erwinia chrysanthemi* strain 3937, which causes soft rot disease on its natural host, *Saintpaulia ionantha*, produces and secretes a battery of depolymerizing enzymes including five isoenzymes of pectate lyase (PL) which can be differentiated by isoelectric focusing (Bertheau *et al.*, 1984). PLs cleave internal glycosidic linkages in pectic polymers by β -elimination. Five *pel* genes organized in two clusters encode these five PL activities (Kotoujansky *et al.*, 1985; Reverchon *et al.*, 1986). The genes *pelB* and *pelC* are adjacent and encode the two 'neutral' isoenzymes, PLb and PLc. One 'acidic' and two 'alkaline' isoenzymes are produced respectively from the *pelA*, *pelD* and *pelE* genes, and form the second cluster. Each gene is transcribed from its own promoter (Diolez & Coleno, 1985; Reverchon *et al.*, 1986), and transcription is induced in the presence of polygalacturonate (PGA).

Despite strong similarity between the PLa and PLe amino acid sequences (Tamaki *et al.*, 1988), PLa shows differences from other secreted PLs of *E. chrysanthemi*. PLa has an acidic isoelectric point, has poor maceration activity on potato tuber (Garibaldi & Bateman, 1971; Barras *et al.*, 1987; Tamaki *et al.*, 1988), and its gene, *pelA*, is hardly induced by PGA (Reverchon *et al.*, 1986). Nevertheless, PLa is essential for full pathogenicity of *E. chrysanthemi* 3937 on *Saintpaulia* plants (Boccara *et al.*, 1988). Furthermore, no cross-reactivity was observed between PLa and others PLs with polyclonal (Pupillo *et al.*, 1976) or monoclonal antibodies (Vergnet-Ballas *et al.*, 1986; Y. Bertheau and others, unpublished).

No antibody against PLa has so far been obtained. To prepare an antiserum which recognizes PLa, the purification of the PLa isoenzyme was undertaken and is presented in this paper. To obtain PLa in its mature state, we chose to purify it from the culture medium of an *E. chrysanthemi* strain overproducing PLa.

Methods

Bacterial strains, plasmids, media and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* strains were grown in Luria broth medium (L medium) (Miller, 1972) at 37 °C. All *Erwinia chrysanthemi* strains used in this study derived from the wild-type strain 3937. *E. chrysanthemi* strains were

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Abbreviations: PGA, polygalacturonate; PL, pectate lyase; PLa, acidic pectate lyase.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M77808.

Table 1. Bacterial strains, plasmids and bacteriophage

Relevant characteristics		Source or reference*
<i>Escherichia coli</i>		
DH5 α	<i>endA1 hsdR17 (r⁻ m⁺) supE44 thi-1 λ^- recA1 gyrA relA1 ϕ80dlacZΔM15 Δ(lacZYA-argF)U169</i>	Bethesda Research Laboratories
<i>Erwinia chrysanthemi</i>		
3937	Wild-type strain isolated from <i>Saintpaulia ionantha</i>	Kotoujansky <i>et al.</i> (1982)
E1006	<i>celZ::\Omega</i> (Spc ^r)	J. L. Aymeric
PMV4122	Δ (<i>pelB pelC pelD pelE pem</i>)	This paper
PMV4123	Δ (<i>pelB pelC pelD pelE pem</i>) <i>celZ::\Omega</i> (Spc ^r)	This paper
PMV4124	PMV4123 derivative containing pPMV0161	This paper
PMV4066	Insertion of Ω fragment in <i>pelA</i> ; Spc ^r	Boccaro <i>et al.</i> (1988)
PMV4125	<i>PelA⁻ PelE⁻</i> (Spc ^r Cm ^r)	M. Boccaro, unpublished
PMV4116	Δ (<i>pel</i>)	F. Renou
Phage		
ϕ EC2	General transducing phage of <i>E. chrysanthemi</i>	Resibois <i>et al.</i> (1984)
Plasmid		
pUBS-3	pUC derivative containing pBLUESCRIPT	G. Murphy
pTZ19R	pUC derivative with T7 RNA polymerase promoter	Mead <i>et al.</i> (1985)
pPMV0161	pTZ19R derivative containing <i>pelA</i> on a <i>DraI</i> fragment of <i>E. chrysanthemi</i> 3937 in the <i>SmaI</i> site (Ap ^r)	This paper

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grown at 30 °C in L medium or in PL-inducing medium (per litre: KH₂PO₄, 3 g; Na₂HPO₄, 0.5 g; MgSO₄·7H₂O, 0.2 g; (NH₄)₂SO₄, 3 g; Casamino acids (Difco), 5 g; polygalacturonic acid (Sigma), 5 g; adjusted to pH 7) (Bertheau *et al.*, 1984). PL-producing bacteria were screened on PGT agar, comprising M9 agar medium (Miller, 1972) supplemented with 5 g glycerol, 1 g yeast extract and 5 g sodium PGA l⁻¹. Antibiotics were used at the following concentrations: ampicillin 50 µg ml⁻¹, chloramphenicol 20 µg ml⁻¹ and spectinomycin 25 µg ml⁻¹.

Preparation of phage lysates. Transduction with phage ϕ EC2 was performed according to Resibois *et al.* (1982).

DNA analysis. Large-scale preparations of plasmid DNAs were made from clear lysates followed by two centrifugations in a caesium chloride/ethidium bromide density gradient (Maniatis *et al.*, 1982). Mini-scale preparations of plasmid DNAs were made from *Escherichia coli* cells by the rapid boiling method of Holmes & Quigley (1981). After restriction, the DNA was electrophoresed in 0.8% agarose gels in Tris/borate buffer (Maniatis *et al.*, 1982). Restriction enzymes and T4 DNA ligase (BRL) were used according to the supplier's recommendations.

DNA sequencing. DNA sequencing was performed by the dideoxy chain-termination method of Sanger *et al.* (1977) using the Sequenase kit (United States Biochemical Corporation). The *pelA*-containing fragment was digested by the restriction enzymes *Sau3A*, *TaqI*, *AluI* and *HpaII*. The resulting fragments were cloned in *Escherichia coli* DH5 α into the plasmid pUBS-3 and used for sequencing. Migration was carried out on gels 80 cm in length (6%, w/v, acrylamide and 460 g urea l⁻¹). Analysis of the sequence was carried out using programs developed by the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

Cup-plate assays. Activities of PLs, proteases, cellulases and pectin methylesterase were tested by the cup-plate method as described by Bertheau *et al.* (1984).

PL assays. PL assays were performed on supernatants of cells grown to late exponential phase in PL-inducing medium. PL activity was

determined by the release from PGA (Sigma) of unsaturated products that absorb at 235 nm (Moran *et al.*, 1968) in 0.1 M-Tris buffer pH 8.6, 5 × 10⁻⁴ M-CaCl₂. One unit of PL activity was defined as 1 µmol unsaturated products liberated min⁻¹ (mg bacterial dry weight)⁻¹.

Preparation of concentrated supernatants. Strain PMV4124 was grown in PL-inducing medium at 30 °C to OD₆₀₀ = 2. The culture was centrifuged at 6000 g and then filtered successively through 8 µm, 0.8 µm, 0.45 µm and 0.22 µm filters (Millipore) and concentrated against polyethylene glycol (Carbowax 20M, Touzard and Matignon) in dialysis tubing. After concentration, the bags were extensively dialysed against 25 mM-imidazole/HCl buffer pH 7.4, for purification, or 100 mM-Tris/HCl buffer pH 8.6, for analytical purposes. A final concentration of 100-fold was achieved. Protein concentration was measured according to Bradford (1976) with bovine serum albumin (Boehringer Mannheim) as a standard. This crude supernatant was stored at 4 °C.

Purification of PLs. Step 1: chromatofocusing. Two millilitres (2 mg protein) of the 100-fold-concentrated crude extract in 25 mM-imidazole/HCl buffer pH 7.4 was loaded onto an HPLC chromatofocusing column (Mono P HR5/20, Pharmacia) equilibrated in the same buffer for 20 min. The adsorbed protein fractions were eluted after 18 min washing, using 55 ml polybuffer 7-4 (Pharmacia) diluted 10-fold and adjusted to pH 3.5. The column was then regenerated with 5 ml 1.5 M-NaCl (flow rate 1 ml min⁻¹). Fractions of 1 ml were collected and PL activity tested by the cup-plate assay. PL-active fractions were pooled, concentrated against polyethylene glycol and dialysed against 100 mM-Tris/HCl buffer pH 8.6.

Step 2: hydrophobic interaction chromatography. The fractions mentioned above were added to an equal volume of 3.4 M-(NH₄)₂SO₄ and loaded onto a hydrophobic interaction HPLC column (Phenyl Superose HR5/5, Pharmacia), equilibrated with 1.7 M-(NH₄)₂SO₄ in 50 mM-Tris/HCl pH 8.6. Elution was done with a decreasing linear gradient of (NH₄)₂SO₄ in the same buffer (flow rate 0.5 ml min⁻¹). The fractions exhibiting PL activity were pooled, further concentrated and dialysed using Centricon 10 microconcentrators (Amicon), and stored at 4 °C.

Isoelectric focusing (IEF). PL isoenzymes were analysed in the supernatant of stationary-phase bacteria grown in PL-inducing medium. Fifteen microlitres of each fraction were layered on a thin polyacrylamide gel and electrofocusing was performed in a pH gradient from 3 to 10 (Pharmacia). PL activities were assessed directly on the gel by an overlay technique according to Bertheau *et al.* (1984).

Polyacrylamide gel electrophoresis. Electrophoresis under denaturing conditions (SDS-PAGE) was performed according to Laemmli (1970), with a 2% (w/v) stacking gel and a 12% (w/v) running gel, using the Mini-Protean II dual slab cell system (Bio-Rad). Gels were fixed and proteins stained according to the silver nitrate method of Merril *et al.* (1981). To enhance subsequent renaturation of PL, bovine serum albumin (Boehringer Mannheim) at 20 µg ml⁻¹ was incorporated in the resolving gel when the gels were to be stained for enzyme activity (Lacks & Springhorn, 1980). The gels were then incubated in several changes of 10 mM-Tris/HCl pH 7.5 for 1 h before being assayed by the overlay technique of Bertheau *et al.* (1984).

Origin and production of antibodies. Polyclonal antibodies were produced in two rabbits by a modification of the method described by Vaitukaitis (1981). The first immunization consisted of 10 intradermal multipoint injections of 100 µl (2.5 µg purified enzyme). Then, on a further four occasions, at 20 d intervals, one subcutaneous injection (1 ml, 25 µg of purified enzyme) was performed. Serum samples were taken just before each injection.

Immunoblotting. Proteins were electrotransferred from SDS-PAGE or IEF gels to nitrocellulose membranes (BA 85, 0.45 µm, Schleicher and Schüll) according to Towbin *et al.* (1979). Proteins bound to the nitrocellulose membrane were stained with 0.5% (w/v) Ponceau red S Electran (BDH) in 5% (w/v) trichloroacetic acid and afterwards washed in water. Immunological detection of proteins on nitrocellulose was slightly modified from Towbin *et al.* (1979). Incubations and rinsing were performed at room temperature. PLa was detected by incubation with the appropriate antisera, followed by goat anti-rabbit IgG alkaline phosphatase conjugate (Biosys). Blots were developed at 37 °C in a mixture (1:1, v/v) of 0.4% (w/v) fast red TR salt (Sigma) in 0.2 M-Tris/HCl buffer pH 8 and a 6% (w/v) solution of naphthol As-Mx (Sigma) in distilled water.

Results

Sequence of the pelA gene

To achieve overexpression of PLa, sequencing of the *pelA* gene of strain 3937 was first undertaken. The *pelA* gene was shown to be included in a 1.6 kb *DraI* fragment, as PLa activity was detected in *Escherichia coli* strains harbouring pPMV0161. This fragment was sequenced. The data, summarized in Fig. 1, revealed a single long open reading frame of 1179 bp, which corresponds to the PLa preprotein. It was not possible to assign putative promoter sequences with assurance, since computer searches failed to disclose any typical *E. coli* promoter sequences 5' to the coding region. However, several putative weak promoter sequences were present. Nuclease S1 mapping experiments have so far been unsuccessful as *pelA* RNA was found to be very unstable (C. Bourson, unpublished). Upstream from the *pelA* gene, we identified two putative KdgR-binding sites.

The KdgR protein regulates many genes involved in the pectin degradation pathway (Condemine & Robert-Baudouy, 1987), and a consensus sequence for KdgR binding showing the typical symmetry of operator sites has been determined (Reverchon *et al.*, 1989; Condemine & Robert-Baudouy, 1991). Furthermore, an AT-rich region, as described for other sequenced *pel* genes (Reverchon *et al.*, 1989; Tamaki *et al.*, 1988), was also observed in the 5' untranslated end of the *pelA* gene. This region could be of importance for the regulation of the *pel* genes. A GC-rich palindromic sequence, followed by a T repeat, occurred after the translational stop codon of *pelA* (TAA). This sequence is typical of rho-independent transcription-termination sites (Von Hippel *et al.*, 1984). All the PL genes of *E. chrysanthemi* so far sequenced possess a TAA stop codon and a rho-independent transcription-termination site (Schoedel & Collmer, 1986; Tamaki *et al.*, 1988; Reverchon *et al.*, 1989).

Upstream of the initiation codon we observed a typical Shine-Dalgarno sequence. The deduced amino acid sequence of the *pelA* gene corresponded to a polypeptide of 393 amino acids, with a calculated molecular mass of 41555 Da, and included a typical N-terminal signal sequence. The cleavage site of this signal peptide was predicted to be between the two alanine residues at nucleotide positions 336 and 337 (Fig. 1). This was confirmed by determining the sequence of the 17 first amino acids of the purified mature protein. The mature PLa protein contains 361 amino acids, and has a calculated molecular mass of 38412 Da and a calculated pI of 5.2. The deduced amino acid sequence of *E. chrysanthemi* 3937 PLa was compared with the published sequences of PLs from *E. chrysanthemi* strains EC16, B374 and 3937. Our sequence has considerable similarity (about 60%) with the PLd and PLc of these three strains and 90% similarity with the PLa of strain EC16. The similarity is greatest in the central part of the protein.

Construction of a strain overproducing PLa

To obtain a strain producing solely PLa, a mutant of strain 3937 deficient in the production of the PLb, PLc, PLd and PLe isoenzymes was constructed by using marker exchange-eviction mutagenesis with the *npt-sacB-sacR* cartridge as described by Ried & Collmer (1988). The clone PMV4122, which had suffered a 6 kb deletion covering the *pelB* and *pelC* genes and a deletion of 6.7 kb which removed the *pelD*, *pelE* and *pem* genes, was selected for PLa production (F. Renou & M. Boccara, unpublished).

The endoglucanase EgZ, secreted by *E. chrysanthemi* 3937, has a pI similar to that of PLa (Boyer *et al.*, 1987). To eliminate EgZ protein, the *celZ*::Ω mutation of strain

DraI 30 50 70 90 110
 GTTACATTTAAAAACATCGTTTCATTATCATCAAAATATTGAAAAACAAATAAATAGATCGTATTTCATTGGCGGCCCTTAAGTGGCGGCATCGCCGAAAACCTTCAATATTAAT

 KdR binding site 150 170 ClaI 210
 AATAAAACATTATTGATAGGTGATGAGGCGTATGGCAGGCAGCCATGAGAGGACGATAAATAAAAAATAACCCATCGATATATATGGAAATTATTTTAAACAATCAACAA

 230 250 270 290 310 330
 AGGATTTTAGTAAGGAAAAATACGATGAACAAAGTATCAGGACGTTCTTTTACCGGCACCTCAACCTGTCTGCTGGCTACATTGATCGCTGGCGTGATGACCTCTGGCGTT

 SD MetAsnLysValSerGlyArgSerPheThrArgThrSerThrCysLeuLeuAlaThrLeuIleAlaGlyValMetThrSerGlyVal
 M N K V S G R S F T R T S T C L L A T L I A G V M T S G V
 350 370 390 410 430
 TCCGCTGCCGAACCTGGTTAAACAGCAAAAGCGCTGGAATCTGCCGCCGCCGCCGCTGGGCGTCAAAAAAGGCTCCACCACCGGCGGTGCCGCTGCAACACGCGATAATAT
 SerAlaAlaGluLeuValAsnSerLysAlaLeuGluSerAlaProAlaAlaGlyTrpAlaSerGlnAsnGlySerThrThrGlyGlyAlaAlaAlaThrSerAspAsnIl
 S A A E L V N S K A L E S A P A A G W A S Q N G S T T G G A A A T S D N I
 450 EcoRI 490 510 530 550
 CTACGCTGTTACCAATATCAGTGAATTTACCCAGCGCGCTCAGCGCCGCCGCCGCTCGCAAAAAATCATCCAGATCACAGGCACGGTCGATATCAGCGCGCGGCACGCCCTACA
 eTyrValValThrAsnIleSerGluPheThrSerAlaLeuSerAlaGlyAlaValAlaLysIleIleGlnIleThrGlyThrValAspIleSerGlyGlyThrProTyrL
 Y V V T N I S E F T S A L S A G A V A K I I Q I T G T V D I S G G T P Y K
 570 590 610 630 650
 AAGATTTTCGCTGATCAGAAAGCCCGCAGCCAGATCAACATTCCGGCTAACACCACCGTGATCGGGATTGGCACCGCAAAATTCATCAACGGCTCTCTGATTATCGAC
 ysAspPheAlaAspGlnLysAlaArgSerGlnIleAsnIleProAlaAsnThrThrValIleGlyIleGlyThrAspAlaLysPheIleAsnGlySerLeuIleIleAsp
 D F A D Q K A R S Q I N I P A N T T V I G I G T D A K F I N G S L I I D
 KpnI 670 690 710 730 750 770
 GGTACCGACGGCACCAATAACGTCATCATCCGCAACGCTCTACATCCAAACGCCAAATTGACGTAGAACCGCATTACGAAAAAGGCGACGGCTGGAACGCCGAATGGGACGG
 GlyThrAspGlyThrAsnAsnValIleIleArgAsnValTyrIleGlnThrProIleAspValGluProHisTyrGluLysGlyAspGlyTrpAsnAlaGluTrpAspGl
 G T D G T N N V I I R N V Y I Q T P I D V E P H Y E K G D G W N A E W D G
 790 SalI 830 850 870
 CATGAACATCACCAACGGTGCCCATCATGTATGGGTGACCCACGTCACCATCAGCGACGGCAGCTTCACCGATGACATGTACACCACCAAAAGCGGCGAAACTTATGTCC
 yMetAsnIleThrAsnGlyAlaHisHisValTrpValAspHisValThrIleSerAspGlySerPheThrAspAspMetTyrThrThrLysAspGlyGluThrTyrValG
 M N I T N G A H H V W V D H V T I S D G S F T D D M Y T T K D G E T Y V Q
 890 910 930 950 970 990
 AGCACGATGGCGCACTGGATATCAAACGCGGTTCCGACTACGTGACCATCTCCAACAGCCTGTTTCGATCAGCACGACAAAAACCATGCTGATTGGTCACAGCGACACCAAC
 lnHisAspGlyAlaLeuAspIleLysArgGlySerAspTyrValThrIleSerAsnSerLeuPheAspGlnHisAspLysThrMetLeuIleGlyHisSerAspThrAsn
 M N G A L D I K R G S D Y V T I S N S L F D Q H D K T M L I G H S D T N
 1010 1030 1050 1070 1090
 TCCGCTCAGGATAAAGGTAAGCTGCACGTCACGCTGTTCAACAAACGTTTAAACCGCGTAACCGAGCGCGCGCGCGCTACGCTACGGCAGCATCCACAGCTTCAACAA
 SerAlaGlnAspLysGlyLysLeuHisValThrLeuPheAsnAsnValPheAsnArgValThrGluArgAlaProArgValArgTyrGlySerIleHisSerPheAsnAs
 S A Q D K G K L H V T L F N N V F N R V T E R A P R V R Y G S I H S F N N
 1110 AclI 1150 1170 1190 1210
 CGTCTTCAACGGTGATGTAAGATACCGGTATACCGTTACCTGTACAGCTTCGGTATCGGCACCGCGGCGAGCGTACTGTCTGAAGGCAACAGCTTCACCATCGCCAAAT
 nValPheAsnGlyAspValLysAspProValTyrArgTyrLeuTyrSerPheGlyIleGlyThrSerGlySerValLeuSerGluGlyAsnSerPheThrIleAlaAsnL
 V F N G D V K D P V Y R Y L Y S F G I G T S G S V L S E G N S F T I A N L
 1230 1250 1270 1290 1310
 TGAGCGCCAGCAAAGCGTGAAGGTTGTGAAGAAATTCACCGGCTCCATTTCTCCGACAAATGGTTCCTGTAATGGCAGCGCGCGCATCTGTCCGGTTGTGGTTTC
 ouSerAlaSerLysAlaCysLysValValLysLysPheAsnGlySerIlePheSerAspAsnGlySerValLeuAsnGlySerAlaAlaAspLeuSerGlyCysGlyPhe
 S A S K A C K V V K K F N G S I F S D N G S V L N G S A A D L S G C G F

Fig. 1. Nucleotide sequence of the 1.6 kb *DraI* fragment containing the *pelA* gene of *E. chrysanthemi* strain 3937. The deduced amino acid sequence of PLA is shown and selected restriction sites are specified. The putative Shine-Dalgarno sequence (SD) and KdgR-binding sites are underlined. The potential rho-independent termination sites are indicated by facing arrows representing the inverted repeat of G + C-rich dyad symmetry preceding the poly(T) sequence. The downward arrow indicates the signal sequence cleavage site of PLA.

E1006 (J. L. Aymeric, unpublished) was introduced into strain PMV4122 by transduction with phage ϕ EC2. A Spc^r CelZ^- transductant was obtained and called PMV4123.

Finally, the 1.6 kb *DraI* fragment containing the *pelA* gene cloned in the *SmaI* site of pTZ19R (Mead *et al.*, 1985) was introduced into strain PMV4123. When the *lacZ* promoter was present upstream of the *pelA* gene, the PLA activity in PL-inducing medium was 15-fold higher than when the *DraI* fragment was in the other orientation. One clone, PMV4124, was retained. The protein profiles after SDS-PAGE of supernatants of strains 3937, PMV4122 and PMV4124 grown in PL-inducing medium (see Fig. 3a) showed that PLA, as identified by its activity, represented the major protein in the culture medium of strain PMV4124 whereas it was hardly visible in the other two strains.

Purification of PLA

Titration curves in polyacrylamide gels showed that PLA had a strong charge variation around its isoelectric point (data not shown). Thus we chose chromatofocusing for the first step of purification.

After chromatofocusing, PL activity appeared in the 42–44 min fractions (Fig. 2a). These fractions were pooled, concentrated and dialysed for the next purification step. No protease, cellulase or pectin methyl-esterase activities were found in these fractions. Silver-nitrate-stained IEF and SDS-PAGE gels showed purity of PLA after this step (Fig. 3a, b). However, the PLA-containing fractions were contaminated with ampholines present in the polybuffer used for elution.

To remove the ampholines, hydrophobic interaction chromatography was used. The ampholines are not fixed onto the hydrophobic gel, whereas the adsorbed PLA activity eluted at the end of the decreasing $(\text{NH}_4)_2\text{SO}_4$ gradient (Fig. 2b). A single protein peak with PLA activity was obtained.

Fig. 2. Chromatography profiles of the purification of PLA. (a) Chromatofocusing profile (Mono P HR5/20) of concentrated PMV4124 culture supernatant. PLA activity is present in the 42–44 min fractions. —, A_{280} ; ---, pH. (b) Hydrophobic interaction chromatography profile. PLA was separated from ampholines on a Phenyl Superose HR5/5 column. PLA activity was found in the 39–42 min fractions. —, A_{280} ; ---, $(\text{NH}_4)_2\text{SO}_4$ concentration.

Fig. 3. Silver-stained SDS-PAGE and IEF gels of samples at different stages of the purification. (a) SDS-PAGE (12% acrylamide; 3 μ g protein loaded). St, molecular mass standards (Bio-Rad); lane 1, concentrated supernatant from strain 3937; lane 2, concentrated supernatant from strain PMV4122; lane 3, concentrated supernatant from strain PMV4124; lane 4, pooled 42–44 min fractions from the chromatofocusing; lane 5, PLA peak (40.2 min) from the hydrophobic interaction chromatography. (b) IEF polyacrylamide gel (pH range 3–10, 5 μ g protein loaded). Lane 1, concentrated supernatant from strain PMV4124; lane 2, PLA peak from the hydrophobic interaction chromatography. The wavy appearance of PLA in IEF gels has often been observed for acidic proteins (Y. Bertheau, unpublished).

Table 2. Recovery of PLA activity and protein during the purification procedure

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Purification (-fold)	PL activity recovered (%)
Supernatant	1000	27.5	28 500	1040	1.0	100
100-fold concentrate	10	12.8	20 270	1580	1.52	71
Mono P HR5/20	2	1.66	3370	2030	1.95	12
Phenyl Superose HR5/5	0.44	1.35	3100	2300	2.21	10.9

Purity of the protein was checked with silver-nitrate-stained SDS-PAGE and IEF gels (Fig. 3*a, b*). Using this two-step purification procedure, PLA was obtained at homogenous purity but with only 11% recovery of PLA activity (Table 2).

Biochemical properties of the PLA

Using a purified preparation of the mature PLA protein isolated from the supernatant fraction of strain PMV4124, the N-terminal residue was shown to be alanine. This classical cleavage site presumably accounts for the fact that PLA is efficiently exported to the periplasm of *Escherichia coli* cells (Reverchon *et al.*, 1985). The purified enzyme migrated in SDS-PAGE

with an apparent molecular mass of 44 kDa. DNA sequence data gave a molecular mass of 39 kDa for the mature protein. This discrepancy was also observed for PLe and PLA of *E. chrysanthemi* EC16 (Tamaki *et al.*, 1988). The behaviour of PLe was previously suspected to be due to its relatively high pI; however, this cannot be applied to PLA. A pI of 4.6 was consistently found in equilibrated IEF gels (Fig. 3*b*), this being different from the value of 5.2 obtained from the DNA sequence and chromatofocusing data. IEF gels and chromatofocusing were performed at different temperatures, at 4 and 20 °C, respectively, which might explain the observed difference in pI values.

An increase in pectin esterification (more than 20% of galacturonic acid residues methoxylated) led to less

Fig. 4. Immunoblots, with a 1000-fold serum dilution, of purified PLa and concentrated culture supernatant from different strains of *E. chrysanthemi* after isoelectrofocusing (1 µg protein). Lane 1, purified PLa from strain PMV4124; lane 2, PMV4122; lane 3, PMV4125; lane 4, PMV4066; lane 5, 3937.

efficient degradation of the substrate by PLa (data not shown). This result was expected, since PLs cleave internal glycosidic linkages by β -elimination after demethylation of the pectic polymer by pectin methyl-esterase. PLa caused a rapid decrease in the viscosity of PGA as measured in an Ostwald viscosimeter with a concomitant production of unsaturated products (a viscosity decrease of 50% corresponds to an A_{235} less than 0.1), suggesting random cleavage of the substrate (data not shown). PLa was most active at pH 8.6 and 50 °C in 0.1 M-Tris/HCl, 5×10^{-4} M- CaCl_2 . The activity was significantly stimulated by Ca^{2+} (5×10^{-4} M- Ca^{2+} increased the activity 15-fold compared to the control without Ca^{2+}), a property which is shared by bacterial and fungal PLs. No other monovalent and divalent cations tested, including Na^+ , K^+ , Mg^{2+} and Mn^{2+} (as chloride salts), stimulated PLa activity. Addition of 0.1 mM-EDTA or 0.5 mM- Hg^{2+} completely inhibited PLa activity. The initial velocity for the reaction allowing the formation of unsaturated oligogalacturonides from PGA, at different concentrations of PGA, in 0.1 M-Tris/HCl buffer pH 8.6, 5×10^{-4} M- CaCl_2 and at 30 °C, was determined by spectrophotometric assay. From a Lineweaver-Burk plot of the data, the apparent K_m and V_{max} were estimated to be 0.43 mg ml $^{-1}$ and 483 U ml $^{-1}$, respectively. These results are similar to

those obtained with PLb and PLc of *E. chrysanthemi* strain CUCPB1237 (Schoedel & Collmer, 1986).

Characterization of antibodies raised against PLa

Polyclonal antibodies were raised against purified PLa. Immunoblotting after IEF of concentrated supernatant (1 µg protein) of strain 3937 revealed two reacting bands at a 1000-fold serum dilution. One band corresponded to PLa (pI 4.6), the other showed a pI value of 9.2, corresponding to the pI of the PLd isoenzyme (Fig. 4). Even at a 200-fold dilution of the serum, no cross-reactivity was observed with PLc, although this PL was shown to have high similarity with PLd (Van Gijsegem, 1989). The band corresponding to PLa was not visible when 1 µg total protein was loaded, but a band corresponding to PLd was detected (not shown). The specificity of our antibodies was confirmed by using concentrated supernatant of strains PMV4066 and PMV4125, which are *pelA* and *pelA pelE* mutants respectively (Fig. 4). In addition, no reacting band was detected with strain PMV4116, a *Pel* $^-$ mutant (data not shown). After SDS-PAGE immunoblotting, only one immunoreactive protein band of 44 kDa was detected in concentrated supernatant of strain 3937 at a 200-fold serum dilution (not shown). The size of this protein

corresponded to that of PLa, PLd and PLe, which exhibited the same molecular mass in SDS-PAGE gels (Fig. 3a).

Discussion

The sequence of the *pelA* gene of *E. chrysanthemi* strain 3937 demonstrated that all the sequences required for the synthesis of the acidic pectate lyase, PLa, were located within a 1.6 kb *DraI* genomic fragment. The deduced amino acid sequences of PL preproteins indicate that their signal peptides are rather long (31 amino acids for PLa of strain 3937, and 30 amino acids for PLe of strains 3937 and EC16), since in prokaryotes, signal sequences are usually 21–23 amino acids long (Von Heijne, 1985). The length of the PLa signal sequence was confirmed by analysing the N-terminal sequence of the purified protein. A signal sequence of 43 amino acids was found for the major endoglucanase EgZ of *E. chrysanthemi* 3937 (Guiseppi *et al.*, 1988). Endoglucanases and PLs are secreted by the same mechanism (Andro *et al.*, 1984), thus a longer signal sequence could play a role in the secretion of these proteins. However, the PLb preprotein possesses a signal sequence of 22 amino acids and PLb is also secreted (Keen & Tamaki, 1986).

In strain 3937, PLa activity represented about 3% of the total PL activity, the *pelA* gene being transcribed at a low level compared to the other *pel* genes (Reverchon & Robert-Baudouy, 1987). So, to obtain an *E. chrysanthemi* strain which overexpresses PLa, the 1.6 kb *DraI* fragment was cloned behind the *lacZ* promoter and used to transform strain PMV4123, which produces only PLa. PLa was the major extracellular protein of the resulting strain PMV4124, which allowed purification of the protein in large amount. To obtain a PL in significant quantities, other groups (Keen & Tamaki, 1986; Schoedel & Collmer, 1986) have previously cloned a *pel* gene on a high-copy-number plasmid in an *Escherichia coli* strain, PL then being purified from the periplasm. The mechanism by which proteins cross the inner membrane seems similar in the two bacteria, but in *E. coli*, PLs are not secreted. Although proteins obtained from the two bacterial species have very similar pI values and molecular masses, one cannot exclude slight modification of the protein after the secretion step, and we therefore chose to purify PLa from the culture supernatant of *Erwinia chrysanthemi* strain PMV4124. In this strain, PLa was the major secreted protein, which might explain the final purification rate of 2.21-fold (Table 2). After the first purification step, 83% of PL activity and 87% of proteins were lost. As PLa is the major supernatant protein (Fig. 2), this loss could be due to

degradation of PLa during its purification, for example by loss of a cofactor.

Biochemical characterization of purified PLa from *E. chrysanthemi* showed that optimal conditions for PLa activity are very similar to those of other *E. chrysanthemi* PLs. PLa activity is stimulated by Ca^{2+} ions, and is very stable. After three years at 4 °C in 100 mM-Tris/HCl buffer pH 8.6, 5×10^{-4} M- Ca^{2+} , the activity was only reduced twofold.

The polyclonal antibodies that we obtained against PLa cross-reacted only with PLd (Fig. 4). Similarly no cross-reactivity was observed between these two isoenzymes by Thurn *et al.* (1987) using polyclonal antibodies raised against PLe from strain EC16, which corresponds to the PLd of strain 3937, or by Vergnet-Ballas *et al.* (1986) using monoclonal antibodies from strain 3937, which recognized solely PLd and PLe of all tested *E. chrysanthemi* strains. It is surprising that the PLe of *E. chrysanthemi* 3937 did not react with our polyclonal antiserum, since PLd is very similar at the sequence level to PLe in the very closely related *E. chrysanthemi* strain B374 (Boccaro *et al.*, 1991; Van Gijsegem *et al.*, 1989), and since the three proteins belong to the same family, according to the homologies observed by Hinton *et al.* (1989). The cross-reactivity observed could be explained by comparing polypeptide sequences, the PLa protein of *E. chrysanthemi* 3937 being slightly more similar to PLd than PLe of strain B374. Alternatively, the three-dimensional structure of PLe could be sufficiently different from those of PLd and PLa to explain these results.

Immunolocalization of PLs secreted by the bacteria in *Saintpaulia* plants infected by *E. chrysanthemi* 3937 has been performed using monoclonal antibodies respectively specific for PLb and PLc (Temsah *et al.*, 1991). The results showed that PLb and PLc were preferentially located in the middle lamella and cell junction, whereas preliminary results indicated that PLd and PLe were preferentially located in the cell wall area close to the plasmalemma (M. Temsah, personal communication). Does PLa act differently *in planta* from the other PLs? Study of the localization of PLa *in planta*, using antibodies, could illuminate the importance of this isoenzyme in pathogenesis. However, monoclonal antibodies seem necessary, since our polyclonal antiserum cross-reacted with PLd. Alternately, a *pelD* mutant could be used.

Different studies (Hinton *et al.*, 1989; Tamaki *et al.*, 1988; Van Gijsegem, 1989; A. Darrasse & Y. Bertheau, unpublished) have shown that the PLa, PLd, PLe family, determined by sequence homology or by PCR amplification, is not present in *Erwinia carotovora* strains. Analysis of the PL profile of a large number of *E. chrysanthemi* strains of different geographical origins has

been performed (Boccarda *et al.*, 1991). PLa was present in most of the strains but not in a very homogeneous group of strains isolated in temperate countries. Studies of PL synthesis of strain 3937 at different temperatures showed an eightfold decrease at 37 °C compared to 30 °C (data not shown). However, strain PMV4122, which secreted only PLa, exhibited the same activity at both temperatures. Taken together, these results might suggest that PLa could contribute to the ability of *E. chrysanthemi* to cause symptoms at supraoptimal temperatures.

The PLa isoenzyme of *E. chrysanthemi* EC16 is a much less efficient macerating factor of plant tissue than PL_e (Garibaldi & Bateman, 1971; Tamaki *et al.*, 1988). It was suggested that the difference in pI between these two proteins may explain this observation. However, results obtained by Tamaki *et al.* (1988) for several recombinant *pelA/pelE* genes suggest that other factors, in addition to the low pI value of PLa, may contribute to its poor macerating efficiency. Despite the poor ability of PLa to macerate potato tubers, Boccarda *et al.* (1988) showed that PLa was essential for full pathogenicity of *E. chrysanthemi* 3937 on *Saintpaulia* plants. PLs with little plant-macerating ability on potato tubers and low pI values, similar to PLa, have also been described for the non-plant-pathogenic bacteria *Klebsiella pneumoniae* and *Yersinia pseudotuberculosis* (Chatterjee *et al.*, 1979; Manulis *et al.*, 1988). The fact that these bacteria were often encountered in the soil raises the possibility that the acidic PLs may have alternative, as yet unknown, physiological functions for the survival of these bacteria in the soil.

Like all the genes of *E. chrysanthemi* involved in pectin degradation that have been identified, *pelA* is regulated by KdgR (C. Bourson, unpublished). Analysis of the *pelA* nucleotide sequence has shown the presence of two putative KdgR-binding sites, one 17 bp long and the other 15 bp long, similar to the KdgR-box consensus sequence A(AT)AAAA(AT)GAAA...TGTTTCAT-T(AT)T(AT)T determined by Condemine & Robert-Baudouy (1991). In PL-inducing medium, *pelA* expression was shown to be very weak compared with that of the other *pel* genes. However, preliminary results suggested that *pelA* is induced by total soluble plant extracts (C. Bourson and others, unpublished). Pupillo *et al.* (1976) have already shown that unlike the other PLs, the induction of PLa was stimulated *in planta*. Studies are in progress to determine whether the conditions for PLa induction differ from those for other PLs; these should give information on the possible role of PLa *in planta*.

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References

- ANDRO, T., CHAMBOST, J. P., KOTOUJANSKY, A., CATTANEO, J., BERTHEAU, Y., BARRAS, F., VAN GISEGEM, F. & COLENO, A. (1984). Mutants of *Erwinia chrysanthemi* defective in secretion of pectinase and cellulase. *Journal of Bacteriology* **160**, 1199–1203.
- BARRAS, F., THURN, K. K. & CHATTERJEE, A. K. (1987). Resolution of four pectate lyase structural genes of *Erwinia chrysanthemi* (EC16) and characterization of the enzymes produced in *Escherichia coli*. *Molecular and General Genetics* **209**, 319–325.
- BERTHEAU, Y., MADGIDI-HERVAN, E., KOTOUJANSKY, A., NGUYEN-THIE, C., ANDRO, T. & COLENO, A. (1984). Detection of depolymerase isoenzymes after electrophoresis or electrofocusing, or in titration curves. *Analytical Biochemistry* **139**, 383–389.
- BOCCARA, M., DIOLEZ, A., ROUVE, M. & KOTOUJANSKY, A. (1988). The role of individual pectate lyase of *Erwinia chrysanthemi* strain 3937 in pathogenicity on *Saintpaulia* plants. *Physiological and Molecular Plant Pathology* **33**, 95–104.
- BOCCARA, M., VEDEL, R., LALO, D., LEBRUN, M. H. & LAFAY, J. F. (1991). Genetic diversity and host range in strains of *Erwinia chrysanthemi*. *Molecular Plant-Microbe Interactions* **4**, 293–299.
- BOYER, M. H., CAMI, B., KOTOUJANSKY, A., CHAMBOST, J. P., FRISON, C. & CATTANEO, J. (1987). Isolation of the gene encoding the major endoglucanase of *Erwinia chrysanthemi*. Homology between *cel* genes of two strains of *Erwinia chrysanthemi*. *FEMS Microbiology Letters* **41**, 351–356.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- CHATTERJEE, A. K., BUCHANAN, G. E., BEHRENS, M. K. & STARR, M. P. (1979). Synthesis and excretion of polygalacturonic acid trans-eliminase in *Erwinia*, *Yersinia* and *Klebsiella* species. *Canadian Journal of Microbiology* **25**, 94–102.
- CONDEMINÉ, G. & ROBERT-BAUDOUY, J. (1987). Tn5 insertion in *kdgR*, a regulatory gene of the polygalacturonate pathway in *E. chrysanthemi*. *FEMS Microbiology Letters* **42**, 39–46.
- CONDEMINÉ, G. & ROBERT-BAUDOUY, J. (1991). Analysis of an *Erwinia chrysanthemi* gene cluster involved in pectin degradation. *Molecular Microbiology* **5**, 2191–2202.
- DEVEREUX, J., HAEERLI, P. & SMITHIES, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* **12**, 387–395.
- DIOLEZ, A. & COLENO, A. (1985). Mu-lac insertion-directed mutagenesis in a pectate lyase gene of *Erwinia chrysanthemi*. *Journal of Bacteriology* **163**, 913–917.
- GARIBALDI, A. & BATEMAN, D. F. (1971). Pectic enzymes produced by *Erwinia chrysanthemi* and their effects on plant tissue. *Physiological Plant Pathology* **1**, 25–40.
- GUISEPPI, A., CAMI, B., AYMERIC, J. L., BALL, G. & CREUZET, N. (1988). Homology between endoglucanase Z of *Erwinia chrysanthemi* and endoglucanases of *Bacillus subtilis* and alkalophilic bacillus. *Molecular Microbiology* **2**, 159–164.
- HINTON, J. C. D., SIDEBOTHAM, J. M., GILL, D. R. & SALMOND, G. P. C. (1989). Extracellular and periplasmic isoenzymes of pectate lyase from *Erwinia carotovora* subspecies *carotovora* belong to different gene families. *Molecular Microbiology* **3**, 1785–1795.

- HOLMES, D. S. & QUIGLEY, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. *Analytical Biochemistry* **114**, 193–197.
- KEEN, N. T. & TAMAKI, S. (1986). Structure of two pectate lyase genes from *Erwinia chrysanthemi* EC16 and their high-level expression in *Escherichia coli*. *Journal of Bacteriology* **168**, 595–606.
- KOTOUJANSKY, A., LEMATTRE, M. & BOISTARD, P. (1982). Utilization of a thermosensitive episome bearing transposon Tn10 to isolate Hfr donor strains of *Erwinia carotovora* subsp. *chrysanthemi*. *Journal of Bacteriology* **150**, 122–131.
- KOTOUJANSKY, A., DIOLEZ, A., BOCCARA, M., BERTHEAU, Y., ANDRO, T. & COLENO, A. (1985). Molecular cloning of *Erwinia chrysanthemi* pectinase and cellulase structural genes. *EMBO Journal* **4**, 781–785.
- LACKS, S. A. & SPRINGHORN, S. S. (1980). Renaturation of enzymes after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. *Journal of Biological Chemistry* **255**, 7467–7473.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MANULIS, S., KOBAYASHI, D. Y. & KEEN, N. T. (1988). Molecular cloning and sequencing of a pectate lyase gene from *Yersinia pseudotuberculosis*. *Journal of Bacteriology* **170**, 1825–1830.
- MEAD, D. A., SKOPURA, E. S. & KEMPER, B. (1985). Single stranded DNA promoter plasmids for engineering mutant RNA's and protein: synthesis of a stretched parathyroid hormone. *Nucleic Acids Research* **13**, 1103–1108.
- MERRIL, C. R., GOLDMAN, D., SEDMAN, S. A. & EBERT, M. H. (1981). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **27**, 1437–1438.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MORAN, F., NASUMO, S. & STARR, M. P. (1968). Extracellular and intracellular polygalacturonic acid trans-eliminases of *Erwinia carotovora*. *Archives of Biochemistry and Biophysics* **123**, 298–306.
- PUPILLO, P., MAZZUCCHI, U. & PIERINI, G. (1976). Pectic lyase isozymes produced by *Erwinia chrysanthemi* Burkh. et al. in polypectate broth or in *Dieffenbachia* leaves. *Physiological Plant Pathology* **9**, 113–120.
- RESIBOIS, A., COLET, M. & TOUSSAINT, A. (1982). Localisation of mini-Mu in its replication intermediates. *EMBO Journal* **1**, 965–969.
- REVERCHON, S. & ROBERT-BAUDOUY, J. (1987). Regulation of expression of pectate lyase genes *pelA*, *pelD*, and *pelE* in *Erwinia chrysanthemi*. *Journal of Bacteriology* **169**, 2417–2423.
- REVERCHON, S., HUGOUVIEUX-COTTE-PATTAT, N. & ROBERT-BAUDOUY, J. (1985). Cloning of genes encoding pectolytic enzymes from a genomic library of the phytopathogenic bacterium, *Erwinia chrysanthemi*. *Gene* **35**, 121–130.
- REVERCHON, S., VAN GISJEGEM, F., ROUVE, M., KOTOUJANSKY, A. & ROBERT-BAUDOUY, J. (1986). Organisation of a pectate lyase gene family in *Erwinia chrysanthemi*. *Gene* **49**, 215–224.
- REVERCHON, S., HUANG, Y., BOURSON, C. & ROBERT-BAUDOUY, J. (1989). Nucleotide sequences of the *Erwinia chrysanthemi* *ogl* and *pelE* genes negatively regulated by the *kdgr* gene product. *Gene* **85**, 125–134.
- RIED, J. F. & COLLMER, A. (1988). Construction and characterization of an *Erwinia chrysanthemi* mutant with directed deletions in all of the pectate lyase structural genes. *Molecular Plant-Microbe Interactions* **1**, 32–38.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.
- SCHOEDL, C. & COLLMER, A. (1986). Evidence of homology between the pectate lyase-encoding *pelB* and *pelC* genes in *Erwinia chrysanthemi*. *Journal of Bacteriology* **167**, 117–123.
- TAMAKI, S. J., GOLD, S., ROBESON, M., MANULIS, S. & KEEN, N. T. (1988). Structure and organization of the *pel* genes from *Erwinia chrysanthemi* EC16. *Journal of Bacteriology* **170**, 3468–3478.
- TEMSAH, M., BERTHEAU, B. & VIAN, B. (1991). Pectate-lyase fixation and pectate disorganization visualized by immunocytochemistry in *Saintpaulia ionantha* infected by *Erwinia chrysanthemi*. *Cell Biology International Reports* **15**, 611–620.
- THURN, K. K., BARRAS, F., KEGOYA-YOSHINO, Y. & CHATTERJEE, A. K. (1987). Pectate lyase of *Erwinia chrysanthemi*: PelE-like polypeptides and *pelE* homologous sequences in strains isolated from different plants. *Physiological and Molecular Plant Pathology* **31**, 429–439.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.
- VAITUKAITIS, J. L. (1981). Production of antisera with small doses of immunogen: multiple intradermal injections. *Methods in Enzymology* **73**, 46–52.
- VAN GISJEGEM, F. (1989). Relationship between the *pel* genes of the *pelADE* cluster of *Erwinia chrysanthemi* strain B374. *Molecular Microbiology* **3**, 1415–1424.
- VERGNET-BALLAS, C., BERTHEAU, Y. & GROSGLAUDE, J. (1986). Production and potential uses of monoclonal antibodies to pectate lyases of *Erwinia chrysanthemi*. *Symbiosis* **2**, 367–372.
- VON HEIJNE, G. (1985). Signal sequences. The limits of variation. *Journal of Molecular Biology* **184**, 99–105.
- VON HIPPEL, P. H., BEAR, D. G., MORGAN, W. D. & MCSWIGGEN, J. A. (1984). Protein-nucleic acid interactions in transcription: a molecular analysis. *Annual Review of Biochemistry* **53**, 389–446.