Proline iminopeptidase gene from *Xanthomonas campestris* pv. *citri*

Jorge Alonso and José L. García

Author for correspondence: José L. García. Tel: +34 1 5611800. Fax: +34 1 5627518.
e-mail: CIBJL16@PINAR1.CSIC.ES

Departamento de
Microbiología Molecular,
Centro de Investigaciones
Biológicas, Consejo
Superior de
Investigaciones Científicas,
Velázquez 144, 28006
Madrid, Spain

The *pip* gene coding for the proline iminopeptidase (Pip) of *Xanthomonas campestris* pv. *citri* was cloned in an *Escherichia coli* *leuB* strain using a selective medium containing the dipeptide D-Ala-L-Leu as the sole source of L-leucine. Nucleotide sequencing of this gene revealed a 939 bp open reading frame encoding a 312 amino acid protein (35 126 Da). The deduced amino acid sequence showed 47% identity with the Pip from *Neisseria gonorrhoeae*. A *lacZ*-*pip* fusion gene was overexpressed in *E. coli* under the control of the *Plac* promoter. The Pip of *X. campestris* hydrolysed L-prolyl-*p*-nitroanilide with the highest efficiency, but was also able to hydrolyse L-alanyl-*p*-nitroanilide and D-alanyl-*p*-nitroanilide. The molecular mass of Pip was found to be 35 kDa by SDS-PAGE and 120 kDa by gel filtration, suggesting that the active enzyme is a multimer.

Keywords: *Xanthomonas campestris* pv. *citri*, proline iminopeptidase, D-alanine

INTRODUCTION

Xanthomonas campestris is a Gram-negative phytopathogenic bacterium belonging to the family *Pseudomonadaceae*. *X. campestris* pv. *citri* is associated with various citrus bacterial diseases (Verniere *et al.*, 1993). Many factors are known to influence the pathogen–plant interaction, and it has been suggested that proteases of phytopathogenic bacteria have a role in disease development (Dow *et al.*, 1993). Protease-deficient mutants of *X. campestris* pathovars induced only mild symptoms and showed reduced bacterial counts in plants (Dow *et al.*, 1993).

Proline iminopeptidase (Pip, EC 3.4.11.5) was reported for the first time by Sarid *et al.* (1959). This enzyme has been found mainly in bacteria (Albertson & Koomey, 1993; Allaker *et al.*, 1994; Atlan *et al.*, 1994; Ehrenfreund *et al.*, 1992; Gilbert *et al.*, 1994; Kitazono *et al.*, 1994a, b; Klein *et al.*, 1994; Yoshimoto *et al.*, 1983) and, to a far lesser extent, in some plants (Ninomiya *et al.*, 1982). Although an activity that cleaves the N-terminal proline of certain proteins has been reported in some mammalian tissues (Hiraoka & Harada, 1991), the presence of a real Pip remains uncertain. Furthermore, some mammalian aminopeptidases, such as leucine aminopeptidases and

carboxylesterases, have a weak activity on the amide substrates used in the Pip assay (Kitazono *et al.*, 1992).

This paper reports the cloning, sequencing and expression in *Escherichia coli* of the *pip* gene from *X. campestris*. Efforts to improve the expression level of this gene, and the characterization of the expressed enzyme, are also described.

METHODS

Bacterial strains, plasmids and media. *X. campestris* pv. *citri* IFO 3835 (Kato *et al.*, 1980a) was provided by the Colección Española de Cultivos Tipo (CECT). *E. coli* strains used were: HB101 [F^- *proA2 leuB supE44 ara-14 galK2 lacY1 Δ(mcrC-mrrK) rpsL20 xyl-5 mtl-1 recA13*] (Sambrook *et al.*, 1989), TG1 [F' (*traD36 lacI^q Δ(lacZ)M15 proA⁺B⁺*) *supE (hsdM-mrcB)5 thi Δ(lac-proAB)*] (Amersham) and GM119 [F^- *dam-3 dcm-6 metB1 supE44 galK2 galT22 lacY1 tsx-78 (thi-1 tonA31 mtl-1)2*] (Arraj & Marinus, 1983). Plasmids pBR322, pUC18 and pUC19 were used for cloning and sequencing. *X. campestris* was grown at 30 °C with shaking in a medium containing 2% (w/v) sucrose, 0.5% peptone, 0.5 g K₂HPO₄ l⁻¹ and 0.25 g MgSO₄·7H₂O l⁻¹ (pH 7.0). *E. coli* cells were cultured in Luria–Bertani broth (Sambrook *et al.*, 1989) at 30 °C or 37 °C with shaking.

Molecular cloning and sequencing procedures. Plasmid DNA was isolated by the alkaline extraction procedure or by CsCl/ethidium bromide equilibrium density gradient centrifugation (Sambrook *et al.*, 1989). *X. campestris* chromosomal DNA was isolated by the method of Chesnney *et al.* (1979). The selected plasmids were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) using double-stranded

Abbreviations: *p*-NA, *p*-nitroanilide; Pip, proline iminopeptidase.

The GenBank accession number for the nucleotide sequence reported in this paper is Z54150.

plasmids as templates and universal or specific oligonucleotides as primers. Southern-blot analysis was carried out as described by Sambrook *et al.* (1989). The DNA probe was labelled using the Polar PLEX Chemiluminescent Blotting Kit (Millipore). Competent cells for transformation were prepared by rubidium chloride treatment (Sambrook *et al.*, 1989). To prepare the genomic libraries of *X. campestris*, the chromosomal DNA was digested with *Bam*HI, *Pst*I, *Hind*III or *Eco*RI and the resulting fragments were ligated to the vector pBR322 previously digested with the corresponding restriction enzyme.

Computer analysis. Nucleotide sequences were analysed using the University of Wisconsin Molecular Biology Package (WIMP) available on the VAX cluster mainframe computer system at the Biotechnology National Center (Madrid). Sequence comparisons were made against the GenBank/EMBL database and the Swissprot database, using the FASTA and TFASTA programs.

Enzyme activity assay. Pip activity was assayed by measuring the amount of *p*-nitroanilide (*p*-NA) liberated from *p*-NA substrates (1 mM) as described by Klein *et al.* (1994). One unit of enzyme activity was defined as the amount of enzyme releasing 1 nmol *p*-NA min⁻¹. The protein concentration was determined by the method of Bradford (1976).

Molecular mass determination. Cell pellets of *E. coli* HB101(pJJ185) were resuspended in 0.1 M Tris/HCl pH 7.5, and disrupted by sonication. The cell suspension was centrifuged at 30000 *g* for 15 min. The clear solution (0.1 ml) was applied to a Superose 12HR 10/30 column (Pharmacia) equilibrated and eluted with 0.1 M Tris/HCl pH 7.5. The fraction showing the highest activity was used for analytical purposes. SDS-PAGE was performed as described by Laemmli (1970). Standard marker proteins were from Bio-Rad.

N-terminal amino acid sequencing. The sample was prepared by applying the purified enzyme solution to a Prosipin sample preparation cartridge containing a PVDF membrane (Immobilon, Millipore). The amino acid sequence was determined by the Edman degradation method using a pulse-liquid sequencer (model 4701; Applied Biosystems).

RESULTS AND DISCUSSION

Isolation and subcloning of the *pip* gene

The *pip* gene of *X. campestris* pv. *citri* IFO 3835 was cloned during the course of a screening programme developed to isolate the *ama* gene encoding the ampicillin acylase (α -amino acid ester hydrolase, ampicillin amidase) of this strain (Kato *et al.*, 1980a). The selection procedure was developed according to a method originally designed to isolate penicillin-acylase-coding genes (García & Buesa, 1986). This method is based upon the auxotrophic complementation of an *E. coli* *leuB* mutant using a minimal medium containing phenylacetyl-L-leucine as the sole source of L-leucine. To clone *ama* genes, we replaced phenylacetyl-L-leucine by D-alanyl-L-leucine in the selective medium, since it has been reported that ampicillin acylase has a broad substrate specificity, being able to hydrolyse different amides and esters of D-amino acids (Kato *et al.*, 1980b).

Among several thousand transformants of *E. coli* HB101 (which is a *leuB* strain) screened from the genomic libraries of *X. campestris* IFO 3835 constructed in pBR322, only one, isolated from a *Pst*I library, was able to grow in

minimal medium using D-alanyl-L-leucine as a source of L-leucine. Since this transformant was unable to grow in the absence of L-leucine we concluded that it was not a *LeuB*⁺ revertant and discarded the possibility that we had cloned the *leuB* gene of *X. campestris*. The isolated plasmid pJJ10 is a pBR322 derivative carrying a 2.8 kb *Pst*I insert (Fig. 1). Southern-blot analyses demonstrated that the cloned fragment was present in the chromosome of *X. campestris* (Fig. 2). Under the same experimental conditions, no hybridization was detected with the *E. coli* chromosome. When competent cells of *E. coli* HB101 were transformed with pJJ10, all the transformants were able to grow in minimal medium containing D-alanyl-L-leucine, suggesting that this plasmid encodes a peptidase that might correspond to the ampicillin acylase activity. Surprisingly, cell extracts prepared from *E. coli* HB101(pJJ10) were unable to hydrolyse ampicillin (data not shown), suggesting either that we had cloned a different gene or that the ampicillin acylase activity produced by this clone was not detected by the current assay used. Moreover, the recombinant was unable to grow in minimal medium containing D-leucyl-L-leucine as a source of L-leucine, indicating a certain specificity for the hydrolysis of the dipeptide.

Nucleotide sequence

To determine the location of the gene encoding the peptidase, the *Pst*I fragment of pJJ10 was digested with different restriction enzymes and subcloned into vectors pUC18 or pUC19, resulting in the plasmids pJJ182, pJJ191, pJJ192, pJJ193, pJJ194 and pJJ195 (Fig. 1). Of these plasmids, only pJJ191 and pJJ195 conferred upon *E. coli* HB101 the ability to grow in the presence of D-alanyl-L-leucine (Fig. 1). Thereafter, plasmid pJJ195 was used to determine the nucleotide sequence of the cloned *Pst*I–*Cla*I fragment (Fig. 3). The sequence revealed the presence of two major overlapping and divergent open reading frames, named ORF1 and ORF2, and a truncated ORF3 (Fig. 3).

Two in-phase ATG codons were found at the 5' terminus of ORF1, but only one was preceded by a sequence (AGGA) resembling a ribosome-binding site (RBS) located at an appropriate distance (Fig. 3). This ORF1 encodes a protein of 312 amino acids (35126 Da) with a calculated pI of 6.4. Two putative promoter regions showing a distance of 21 bp between the respective –35 and –10 boxes and a low similarity with the *E. coli* σ^{70} consensus promoter were detected upstream of this ORF1 (Fig. 3). Although a distance of 21 bp between boxes and a low consensus similarity have been observed in known promoters (Hawley & McClure, 1983), the precise transcription start site remains to be elucidated. Furthermore, there were no palindromic sequences suggestive of a terminator structure downstream of the TAA stop codon of ORF1. A database search showed that the putative protein encoded by ORF1 was highly similar (47% identity, 67% similarity) to the Pip enzyme from *Neisseria gonorrhoeae*. This protein also shows a limited similarity with other Pips and hydrolases of aromatic compounds, e.g. Pip from *Aeromonas sobria* (24% identity, 54%

Fig. 1. Restriction map of the *pip* gene from *X. campestris*. Abbreviations: C, *ClaI*; C*, methylated *ClaI* site; M, *MaeI*; P, *PstI*; S, *SalI*. Enzyme specific activity [U (mg protein)⁻¹] was determined in extracts of the *E. coli* HB101 recombinants using L-prolyl-*p*-NA as substrate (ND, not detected). In pJJ185 the *pip* gene is in-frame with *lacZ* on the vector.

similarity), Pip from *Lactobacillus delbrueckii* (24 % identity, 51 % similarity), Pip from *Bacillus coagulans* (24 % identity, 47 % similarity), 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase from *Pseudomonas putida* (26 % identity, 49 % similarity), 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase from *P. putida* (25 % identity, 49 % similarity) and 2-hydroxymuconic semialdehyde hydrolase from *P. putida* (23 % identity, 46 % similarity). This similarity is especially marked in the regions surrounding the postulated catalytic amino acids (Díaz & Timmis, 1995; Kitazono *et al.*, 1994b) (Fig. 4).

ORF2 and ORF3 did not have any RBS-like sequences preceding the respective start codons, and their deduced amino acid sequences did not show any significant similarity with other proteins contained in the databanks.

Expression of the cloned gene and identification of its product

The findings reported above strongly suggested that we had cloned the *pip* gene of *X. campestris* pv. *citri*. To confirm this, cell extracts of *E. coli* cells harbouring the subcloned fragments were analysed for the presence of a Pip-like activity. A Pip activity was only detected in the

recombinants able to grow in the presence of D-alanyl-L-leucine, carrying the complete *pip* gene (Fig. 1). This result demonstrated that the hydrolytic activity was not due to the Pip of *E. coli* and strongly suggested that it can be ascribed to the cloned gene.

Since the production of Pip in *E. coli* HB101(pJJ195) was very low and the *pip* expression was not inducible by IPTG (data not shown) we assumed that the gene was transcribed under the control of a weak promoter contained in the cloned fragment. To express the *pip* gene under the control of an *E. coli* promoter we isolated the ORF1 using the restriction sites *MaeI* and *ClaI* located in its noncoding flanking regions. To allow the digestion of the methylated *ClaI* site, pJJ195 was isolated from *E. coli* GM119. Thereafter, the 1 kb *MaeI*–*ClaI* fragment carrying the *pip* gene was made blunt-ended, purified and ligated to *SmaI*-digested pUC18. This construction generates a stop codon just at the *SmaI*–*MaeI* junction that avoids the creation of an in-frame fusion with the *lacZ* gene of pUC18. One of the resulting plasmids, named pJJ186 (Fig. 1) was sequenced to confirm the accuracy of the construction. Interestingly, among the isolated *E. coli* HB101 recombinant clones, two transformants displayed a very high Pip activity on L-prolyl-*p*-NA measured in cell

Fig. 2. Southern hybridization of chromosomal DNA from *X. campestris*. The filter was probed using the 2.8 kb *Pst*I fragment from pJJ10. Lane 1, *E. coli* HB101 DNA digested with *Pst*I; lane 2, *X. campestris* DNA digested with *Pst*I; lane 3, λ DNA digested with *Bst*EII; lane 4, 2.8 kb *Pst*I fragment from pJJ10; lane 5, 1.0 kb *Mae*I–*Cla*I fragment from pJJ195.

extracts. These clones harboured a similar, but not identical, plasmid, named pJJ185, which had lost a nucleotide in the *Sma*I–*Mae*I junction, producing an in-frame fusion with the *lacZ* gene of the vector. Cells harbouring pJJ185 hyperproduced a 37 kDa protein that corresponds to the expected size of the fusion protein (Fig. 5). Due to the high amount of Pip produced by *E. coli* HB101(pJJ185) the enzyme can be purified in one step by gel filtration (Fig. 5). The N-terminal amino acid sequence, Thr-Met-Ile-Thr-Asn, found in the purified protein perfectly matched the predicted β -galactosidase–Pip fusion. The production of the fusion protein was inducible by IPTG in *E. coli* TG1 and constitutive in HB101, indicating that *pip* expression is under the control of the *lac* promoter (Fig. 5). The Pip activity produced by clones harbouring pJJ185 was 40-fold higher than that produced by cells carrying pJJ186 and 400-fold higher than that detected in *X. campestris* (Fig. 1). The high production of the Pip fusion can probably be ascribed to a higher initiation efficiency (MacCarthy & Brimacombe, 1994). Interestingly, a similar result was observed with the prolyl aminopeptidase from *Aeromonas sobria*, where a fusion protein with the β -galactosidase produced a 19-fold increase in enzyme activity (Kitazono *et al.*, 1994b).

Characterization of the Pip activity

Pip prepared from *E. coli* TG1(pJJ186) behaved in gel filtration as a protein with an apparent molecular mass of 120 kDa, suggesting that the active enzyme might be a tetramer, as already suggested for the Pip of *Aeromonas sobria* (Kitazono *et al.*, 1994b) or apricot seeds (Ninomiya

Fig. 3. Nucleotide sequence of the *pip* gene from *X. campestris* *pv. citri* and its deduced amino acid sequence. The putative RBS sequence (AGGA) and sequences that resemble the strong promoters of *E. coli* are underlined. The initiation and stop codon are shown in bold letters. Arrows indicate the direction of transcription of the ORFs.

et al., 1982), but we cannot completely rule out the possibility that it might be a trimer having a non-globular shape like the Pip enzyme from *Lactobacillus delbrueckii* subsp. *bulgaricus* (Gilbert *et al.*, 1994). The optimum pH for the enzyme was 7.5 and the maximum activity was observed at 40 °C. The fusion protein prepared from *E. coli* TG1(pJJ185) also behaved in gel filtration as a multimer showing an apparent molecular mass of 150 kDa. Recently, this fusion protein has been crystallized and the preliminary diffraction data suggest that its three-dimensional structure can probably be solved soon (F. J. Medrano, personal communication).

The results shown in Table 1 indicate that the Pip of *X. campestris* hydrolysed L-prolyl-p-NA with the highest

A			B			C		
(1)	99	LGVDRW-QVFGG SWG STLALAYAADPSAAGHQLVLRG	134	256	PGVIVHGRYDVVCPQSAWDLHKAWPERQLQISPASGHS-A-FEPENVDAIVR	306		
(2)	96	LGIGKW-LVFGG SWG STLSLAYAQTHPERVKLLVLRG	131	251	PTVIVQGRYDLCTFMQSAWELSKAFPEALRVVQA-GHCAAFDPPLADALVQ	301		
(3)	90	LNLDEV-HILGH SWG TTLAAAY-CLTKPSGVKSVIFS	124	231	PSLYTCGRFDEATPETTEY-YSSLTPKSKFHVFEKSAHMP-YIEEPPEYLAV	279		
(4)	95	LGLDQI-HLLG SWG GMLALIYLCDYQPKGVKSLILS	130	236	PALITSGTDDLCTPLVAKS-MYDHLPNARWELFAGCGHMP-FVQENAKYQEL	284		
(5)	134	LSPDHPWSLLG SWG FGGFCSTYLSLFPDSLHEVYLTG	170	367	PVACAVYAEDMYVEFDYSRETLKGLSNSRAWITNEYENGLRVLDGEQILDRL	417		
(6)	94	LELDRV-DLVGNSFGGALSLAFIRFPHRVRLVIMG	129	217	ETLILHGRDDRVIPLTSLRLNQLIEPSQLHVFGRCGHVW-QIEQNRGFIRL	266		
(7)	95	LGIQGG-DIVGNSFGGGLALALAIRHPERVRLVIMG	130	218	ETLVHGREDDRIPLQASLTIAQWIPNAQLHVFGCGHWT-QIEHAERFARL	267		
(8)	101	LGIDRA-HLVGNSMGGATALNFAIEYFDRIGKLILMG	136	228	KTFITWGRDDRFPVLDHGLKLLWNIDDARLHVFSKCGHWA-QWEHADEFNRL	277		
(9)	99	MGLHNT-TVIGH SWG MTAGVLASIHDPKVSRLVLIS	134	216	KTLLWGNQNPMTESMQNDIRALPKAKFIQYNGFGHSM-FWEDPEMVAKD	265		

Fig. 4. Alignment of the regions surrounding the catalytic amino acids of several enzymes. (1), Pip from *X. campestris* (this work); (2), Pip from *N. gonorrhoeae* (Albertson & Koomey, 1993); (3), Pip from *B. coagulans* (Kitazono *et al.*, 1992); (4), Pip from *L. delbrueckii* (Klein *et al.*, 1994); (5), Pip from *A. sobria* (Kitazono *et al.*, 1994a); (6), 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase from *P. putida* (Menn *et al.*, 1991); (7), 2-hydroxymuconic semialdehyde hydrolase from *P. putida* (Horn *et al.*, 1991); (8), 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase from *P. putida* (Hayase *et al.*, 1990); (9), atropinesterase from *P. putida* (Hessing, 1983). A, B and C indicate the regions surrounding the postulated catalytic amino acids that are shown in bold letters.

Fig. 5. SDS-PAGE of bacterial lysates of *E. coli* transformants. Each lysate and the purified enzyme were electrophoresed in a 10% (w/v) polyacrylamide gel and stained with Coomassie Brilliant Blue. Lane 1, TG1(pUC18); lane 2, TG1(pUC18) + 1 mM IPTG; lane 3, TG1(pJJ185); lane 4, TG1(pJJ185) + 1 mM IPTG; lane 5, TG1(pJJ186); lane 6, TG1(pJJ186) + 1 mM IPTG; lane 7, HB101(pJJ185); lane 8, molecular mass standards; lane 9, LacZ-Pip fusion protein purified by gel filtration.

Table 1. Hydrolysis of various substrates by the Pip from *X. campestris*

Substrate	Activity [U (mg protein) ⁻¹]*			
	<i>E. coli</i> HB101 extracts			Purified fusion
	(pUC18)	(pJJ186)	(pJJ185)	
L-Prolyl- <i>p</i> -NA	ND	47	2251	7484
L-Alanyl- <i>p</i> -NA	80	117	1948	6237
D-Alanyl- <i>p</i> -NA	ND	1	20	66

* The values represent the means of three independent experiments, performed with cell extracts of *E. coli* HB101 recombinants carrying plasmids pUC18 (control), pJJ186 (wild-type Pip) or pJJ185 (Pip fusion) or the purified Pip fusion. ND, Not detected. None of the four preparations showed detectable activity against L-phenylalanyl-*p*-NA, D-phenylalanyl-*p*-NA, L-leucyl-*p*-NA or D-leucyl-*p*-NA.

efficiency (100% activity), but it was also able to hydrolyse L-alanyl-*p*-NA (80% activity) and D-alanyl-*p*-NA (1% activity). Interestingly, L-alanyl-*p*-NA was not efficiently

cleaved by the Pip of two subspecies of *L. delbrueckii* (Gilbert *et al.*, 1994; Klein *et al.*, 1994). The Pip from apricot seed hydrolyses glycyl- β -naphthylamide at a low efficiency but not the L-alanine derivative (Ninomiya *et al.*, 1982). The finding that D-alanyl-*p*-NA can be hydrolysed at low efficiency by the Pip of *X. campestris* probably explains why the D-alanyl-L-leucine dipeptide is used *in vivo* by *E. coli* HB101(pJJ10) as a source of L-leucine by the *pip* recombinants, and fits with the observation that this dipeptide was hydrolysed *in vitro* at a low but detectable rate (data not shown). In contrast, the dipeptide D-leucyl-L-leucine cannot be used *in vivo* by *E. coli* HB101(pJJ10) as a source of L-leucine and the D-leucyl-*p*-NA cannot be hydrolysed *in vitro*.

Concluding remarks

The very low endogenous Pip activity of *E. coli* has allowed its use as a host for cloning and expressing the *pip* gene from *X. campestris* pv. *citri*. The substrate specificity of this novel Pip provides a formal explanation for the fortuitous isolation of its encoding gene using a selection method that was initially developed to clone a different

family of genes. The enzyme was produced as a soluble protein in *E. coli* and *X. campestris*. The Pip activity was not detected in the culture medium and it is not produced as a preprotein. The Pip characterized in this work is not the proline-specific endopeptidase previously described in *Xanthomonas* sp. (Szwajcer-Dey *et al.*, 1992), since this endopeptidase is a monomer with a molecular mass of 75 kDa and has a different substrate specificity.

Finally, it is worth noting that the Pip has been suggested to be a virulence factor of some human pathogenic bacteria such as *Eikenella corrodens* (Allaker *et al.*, 1994) and *N. gonorrhoeae* (Albertson & Koomey 1993). Hence, based on the observation that protease-deficient mutants of *X. campestris* pathovars showed a reduced virulence (Dow *et al.*, 1993), it is possible that this enzyme might influence the capacity of *X. campestris* to cause plant damage.

ACKNOWLEDGEMENTS

We are grateful to F. J. Medrano, P. García, R. López and E. García for valuable suggestions and discussions. The technical assistance of M. Carrasco and E. Cano is gratefully acknowledged. We also wish to thank A. Hurtado for the artwork.

REFERENCES

- Albertson, N. H., & Koomey, M. (1993). Molecular cloning and characterization of a proline iminopeptidase gene from *Neisseria gonorrhoeae*. *Mol Microbiol* **9**, 1203–1211.
- Allaker, R. P., Young, K. A. & Hardie, J. M. (1994). Production of hydrolytic enzymes by oral isolates of *Eikenella corrodens*. *FEMS Microbiol Lett* **123**, 69–74.
- Atlan, D., Gilbert, C., Blanc, B. & Portalier, R. (1994). Cloning, sequencing and characterization of the *pepIP* gene encoding a proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397. *Microbiology* **140**, 527–535.
- Arraj, J. A. & Marinus, M.-G. (1983). Phenotypic reversal in *dam* mutants of *Escherichia coli* K-12 by recombinant plasmid containing the *dam*⁺ gene. *J Bacteriol* **153**, 562–565.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Chesney, R. H., Scott, J. R. & Vapnek, O. (1979). Integration of the plasmid prophages P1 and P7 into the chromosome of *Escherichia coli*. *J Mol Biol* **130**, 161–173.
- Díaz, E. & Timmis, K. N. (1995). Identification of functional residues in a 2-hydroxymuconic semialdehyde hydrolase. *J. Biol Chem* **270**, 1–9.
- Dow, J. M., Fan, M. J., Newman, M.-A. & Daniels, M. J. (1993). Differential expression of conserved protease genes in crucifer-attacking pathovars of *Xanthomonas campestris*. *Appl Environ Microbiol* **59**, 3996–4003.
- Ehrenfreund, P., Mollay, C. & Kreil, G. (1992). Purification and properties of an iminopeptidase from culture media of *Streptomyces plicatus*. *Biochem Biophys Res Commun* **184**, 1250–1255.
- García, J. L. & Buesa, J. M. (1986). An improved method to clone penicillin acylase genes: cloning and expression in *Escherichia coli* of penicillin G acylase from *Kluyvera citrophila*. *J Biotechnol* **3**, 187–195.
- Gilbert, C., Atlan, D., Blanc, B. & Portalier, R. (1994). Proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397: purification and characterization. *Microbiology* **140**, 537–542.
- Hawley, D. K. & McClure, W. R. (1983). Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Res* **11**, 2237–2255.
- Hayase, N., Taira, K. & Furukawa, K. (1990). *Pseudomonas putida* KF715 *bphABCD* operon encoding biphenyl and polychlorinated biphenyl degradation: cloning, analysis and expression in soil bacteria. *J Bacteriol* **172**, 1160–1164.
- Hessing, J. G. M. (1983). *The primary structure of atropinesterase from Pseudomonas putida*. PhD thesis, University of Leiden.
- Hiraoka, B. & Harada, M. (1991). Use of L-prolyl-leucylglycinamide (MIF-1) for the high-performance liquid chromatographic determination of proline iminopeptidase activity in rat liver. *J Chromatogr* **563**, 142–146.
- Horn, J. M., Harayama, S. & Timmis, K. N. (1991). DNA sequence determination of the TOL plasmid (pWW0) *xylIGFJ* genes of *Pseudomonas putida*: implications for the evolution of aromatic catabolism. *Mol Microbiol* **5**, 2459–2474.
- Kato, K., Kawahara, K., Takahashi, T. & Kakinum, A. (1980a). Purification of α -amino acid ester hydrolase from *Xanthomonas citri*. *Agric Biol Chem* **44**, 1069–1074.
- Kato, K., Kawahara, K., Takahashi, T. & Kakinum, A. (1980b). Substrate specificity of α -amino acid ester hydrolase from *Xanthomonas citri*. *Agric Biol Chem* **44**, 1075–1081.
- Kitazono, A., Yoshimoto, T. & Tsuru, D. (1992). Cloning, sequencing and high expression of the proline iminopeptidase gene from *Bacillus coagulans*. *J Bacteriol* **174**, 7919–7925.
- Kitazono, A., Kitano, A., Tsuru, D. & Yoshimoto, T. (1994a). Isolation and characterization of the prolyl aminopeptidase gene (*pap*) from *Aeromonas sobria*: comparison with the *Bacillus coagulans* enzyme. *J Biochem* **116**, 818–825.
- Kitazono, A., Ito, K. & Yoshimoto, T. (1994b). Prolyl aminopeptidase is not a sulfhydryl enzyme: identification of the active serine residue by site-directed mutagenesis. *J Biochem* **116**, 943–945.
- Klein, J. R., Schmidt, U. & Plapp, R. (1994). Cloning, heterologous expression, and sequencing of a novel proline iminopeptidase gene, *pepI*, from *Lactobacillus delbrueckii* subsp. *lactis* DSM 7290. *Microbiology* **140**, 1133–1139.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- MacCarthy, J. E. G. & Brimacombe, R. (1994). Prokaryotic translation: the interactive pathway leading to initiation. *Trends Genet* **10**, 402–407.
- Menn, F. M., Gerben, J. Z. & Gibson, D. T. (1991). Location and sequence of the *todF* gene encoding 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase in *Pseudomonas putida*. *Gene* **104**, 91–94.
- Ninomiya, K., Kawatani, K., Tanaka, S., Kawata, S. & Makisumi, S. (1982). Purification and properties of a proline iminopeptidase from apricot seeds. *J Biochem* **92**, 413–421.
- Sambrook, J., Fritsch, E. J. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Sarid, S., Berger, A. & Katchalski, E. (1959). Proline iminopeptidase. *J Biol Chem* **234**, 1740–1744.
- Szwajcer-Dey, E., Rasmussen, J., Meldal, M. & Breddman, K. (1992). Proline-specific endopeptidases from microbial sources: isolation of an enzyme from *Xanthomonas* sp. *J Bacteriol* **174**, 2454–2459.
- Verniere, C., Provost, O., Civerolo, E. L., Gambin, O., Jacquemoud-

Collet, J. P. & Luisette, J. (1993). Evaluation of the biolog substrate utilization system to identify and assess metabolic variation among strains of *Xanthomonas campestris* pv. citri. *Appl Environ Microbiol* **59**, 243–249.

Yoshimoto, T., Saeki, T. & Tsuru, D. (1983). Proline iminopeptidase

from *Bacillus megaterium*: purification and characterization. *J Biochem* **93**, 469–477.

Received 16 April 1996; revised 27 June 1996; accepted 8 July 1996.