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Isolation and characterization of new C-terminal substitution mutations affecting secretion of polygalacturonase in *Erwinia carotovora* ssp. *carotovora*

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Abstract An intact C-terminus was previously shown to be required for stability and secretion of the polygalacturonase (PehA) in *Erwinia carotovora* ssp. *carotovora*. Here we have analyzed the effects of amino acid (aa) substitutions generated to five C-terminal positions of PehA. Conservation of two hydrophobic and one non-hydrophobic residue (V372, V374 and N371, respectively) was found to be essential for maintenance of the protein stability. As an exception, one of the mutants (V372G) did not show major effects on protein stability, as determined by immunoblots and enzyme activity assay, yet it prevented the secretion completely. We conclude that the C-terminus of PehA is directly involved in the formation or stabilization of a conformation-sensitive structure needed for recognition of the protein as secreted.

Key words: Gram-negative; Protein secretion; Polygalacturonase; Mutagenesis; Secretion-deficient mutant

1. Introduction

The plant pathogenic bacterium *Erwinia carotovora* ssp. *carotovora* (*Ecc*) causes soft-rot disease in various crop plants by secreting plant cell wall degrading enzymes, such as pectate lyase, pectin lyase, polygalacturonase (Peh), cellulase and protease [1,2]. Production and secretion of these extracellular enzymes are essential for virulence, since mutants defective either in their production (*exp, aep* and *rex* mutations) or secretion (*out* mutations) are avirulent or exhibit a reduced virulence [3–6]. Pectinases and cellulases are secreted through the general secretion (GSP) [7] or type II secretion pathway [8] which is a central mechanism for targeting extracellular proteins in Gram-negative bacteria. Secretion through the *Ecc* Out pathway requires at least 13 accessory proteins that are considerably conserved with the corresponding components in other bacteria, such as *E. chrysanthemi, Klebsiella, Xanthomonas* and *Aeromonas* [9]. Secretion is a two-step process where the proteins are first translocated in a signal peptide dependent manner across the cytoplasmic membrane by the Sec system [10,11]. Next, the proteins must fold in their final conformation in the periplasm before they are routed further across the outer membrane and finally released into the extracellular milieu [12–14].

Secreted proteins are supposed to possess another targeting signal in their mature amino acid sequence that is recognized by the components of the secretion apparatus. Regions containing information needed for secretion have been localized in aerolysin of *Aeromonas salmonicida* [15] and exotoxin A of *Pseudomonas aeruginosa* [16,17]. Lu and Lory [17] showed that a segment between amino acids 60 and 120 is needed for translocation across the outer membrane. Interestingly, McVay and Hamood conclude that even the C-terminus of exotoxin A would include similar information [18]. Thus, the protein would contain several, separate targeting signals. Py et al. [13] suggested that information needed for secretion of the cellulase EGZ of *E. chrysanthemi* could lie in several parts of the protein, yet in this case the separate regions were not independently able to promote secretion, but rather they were thought to form a single targeting signal. Also, since EGZ has to form disulfide bonds in the periplasm before secretion, and the region flanking a bond-forming cysteine shares similarity with the corresponding region in two pectinases, this region could contain information relevant for secretion [14].

Heterologous proteins can be routed out of the cell via GSP using the targeting information of a secretory protein. Kornacker and Pugsley [19] showed that the N-terminal two-thirds of *Klebsiella oxytoca* pullulanase (Pul) was sufficient to direct secretion of the fused passenger molecule, β-lactamase (Bla). On the other hand, corresponding PehA-Bla hybrid proteins remained cell-bound [6]. Recently, we showed that the C-terminus of PehA contains information related to secretion, and e.g. PehA-Bla hybrid proteins can specifically be secreted by the *Ecc* GSP, provided that no more than the last two aa of the PehA domain were removed from the fusion [20]. To analyze in detail the relevance of the C-terminus of PehA for correct extracellular targeting we have here created defined substitutions to five terminal residues and characterized the properties of the mutant proteins in *Ecc*.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in the study are listed in Table 1. *E. coli* and *Ecc* cultures were grown in L-medium [21] in a rotary shaker at 37 and 28°C, respectively. Ampicillin (Ap) was added at 150 μg ml^{-1}. For protein analysis and enzymatic assays in *Ecc*, the cultures were supplemented with 0.4% glycerol and grown to stationary phase, diluted at 1:100 and grown further for 20 h before collecting samples.

2.2. Genetic techniques

E. coli cells were made competent and transformed according to Maniatis et al. [22]. Electroporation of *Ecc* cells was performed as described previously [20].

2.3. DNA techniques

Plasmid DNA was isolated by alkaline lysis according to Birnboim and Doly [23]. Restriction enzymes, T4 ligase, T4 polynucleotide ki-

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nase and alkaline phosphatase were used according to the manufacturer's instructions (Promega, Madison, WI, USA). DNA fragments were separated by agarose-gel electrophoresis [22]. Site-directed mutagenesis of the *pehA* gene was performed with polymerase chain reaction (PCR) using degenerate oligonucleotides. PCR was performed with the following oligonucleotides: no. 9512, TA ACG GAT CCA AGC ATA AAG CCC AGT GCT TTT hybridized to the *pehA* promoter region at 194–172 nt upstream from the translation start site. no. 949, TA AGG ATC CTA CTT CTT GNC (G/C)T(A/T/G) GAC GTT C; no. 9414, CA TGG ATC CTA CTT CTT GAN GTT GAC G; no. 9415, CA TGG ATC CTA CTT CTT GAC GTT GNC GTT CT; no. 9518, CA TGG ATC CTA CTT CTT GAC GTT GAC GTT CNT GAT TTG CC; no. 9519, CA TGG ATC CTA CTT CTT GAC GTT GAC GNT CTT GAT TTG CC; hybridized to the 3′ end of the coding sequence of *pehA* and read towards the gene. The following changes in the aa sequence were created: no. 949, N373→Y/H and V374→A/D/G; no. 9414, V374→L/I/F; no. 9415, V372→A/D/G; no. 9518, K370→T/R/M; no. 9519, N371→S/T/I. DNA was amplified for 20 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 2 min, using *Pfu* polymerase (Stratagene, La Jolla, CA, USA). Mutated *pehA* fragments were cloned in pUC18 at the *Bam*HI or *Sma*I site, and the plasmids were propagated in *E. coli* followed by transfer to *Ecc*. DNA sequence analysis was performed from plasmids employing the dideoxy chain-termination method of Sanger et al. [24] using a Sequenase quick-denature plasmid sequencing kit (US Biochemicals, Cleveland, OH). The following oligonucleotide, TC CCA GAC TGG AGT GAT ATC, hybridizing in the *pehA* gene at 1043–1062 nt downstream from the translation start point, was used as a primer in sequencing.

2.4. Protein analysis

SDS-PAGE was performed in 12% polyacrylamide gels according to Laemmli [25] and transfer of proteins to nitrocellulose filters according to Burnette [26]. Proteins were detected with a polyclonal rabbit anti-PehA antiserum and an alkaline phosphatase-conjugated second antibody as described [20]. Periplasmic proteins were isolated by osmotic shock treatment as described [27], except that in the final step the periplasmic proteins were recovered in 20 mM $MgCl_2$.

2.5. Enzyme assays

Polygalacturonase [28] and β-lactamase [29] activities were assayed as described.

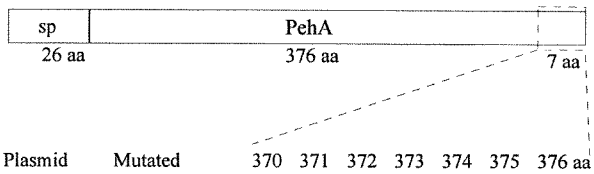
3. Results

PCR primers used to generate base substitutions in the 3′ end of the *pehA* gene allowed mutagenesis of one or two codons at a time. The mutated *pehA* variants were amplified in the pUC18 plasmid in *E. coli* and individual mutations were verified by DNA sequencing. The deduced amino acid changes of the various mutants are shown in Fig. 1. To ensure that no undesired second site mutations were generated during PCR, two to three mutant plasmids of each type were initially analyzed in *Ecc*. Thereafter, one representative of each type was chosen for further analysis.

The C-terminus of PehA contains two hydrophobic residues, V372 and V374. Upon expression in *Ecc*, the V374D substitution was formerly found to cause severe effects on both secretion and protein stability while V374A led to minor effects ([20], Fig. 2A, lanes 2,3). During closer analysis of mutations affecting this position we observed that a flanking N373Y substitution could reverse the effects caused by V374D. The double mutant N373Y-V374D exhibited nearly wild-type levels of secretion and stability (Fig. 2B, lane 3). Consistent with the immunoblot analysis, the distribution and level of Peh activity were also close to those of the wild-type (Table 2). Apparently, the large aromatic ring of tyrosine was able to suppress the effects exerted by the neighboring residue, suggesting that hydrophobicity as such, rather than a specific residue, might be required for the position. To test this hypothesis, the hydrophobic aa Leu, Ile and Phe were

Table 1
Bacterial strains and plasmids

Bacterial strain	Genotype	Origin/reference
E. coli K12		
HB101	F⁻ *thi-1 hsdS20* (r^-m^-) *supE44 recA13 ara-14 leuB6 proA2 lacY1 rpsL20 xyl-5 mtl-1*	[31]
DH5α	F⁻ Δ(*lacZYA-argF) recA endA1 gyrA1 thi1 hsdR17 supE44 relA1 (ϕ80d lacZΔM15*)	Bethesda Research Laboratories
E. carotovora ssp. *carotovora*		
SCC3193	wild type	[32]
HSE2201	SCC3193 *pehA*::Tn*1731*	[20]
Plasmid		
pUC18	Ap^R, cloning vector	[33]
pHSK24	Ap^R, *pehA*	[34]
pTPE1	$pehA^{wt}$ in pUC18	[20]
pTPE7	*pehA* (V374A) in pUC18	[20]
pTPE8	*pehA* (V374D) in pUC18	[20]
pTPE13	*pehA* (N373Y) in pUC18	[20]
pTPE14	*pehA* (N373Y-V374A) in pUC18	This work
pTPE15	*pehA* (N373Y-V374D) in pUC18	This work
pTPE16-1	*pehA* (V372A) in pUC18	This work
pTPE16-2	*pehA* (V372D) in pUC18	This work
pTPE16-3	*pehA* (V372G) in pUC18	This work
pTPE17-1	*pehA* (V374L) in pUC18	This work
pTPE17-2	*pehA* (V374I) in pUC18	This work
pTPE17-3	*pehA* (V374F) in pUC18	This work
pTPE18-1	*pehA* (N371S) in pUC18	This work
pTPE18-2	*pehA* (N371T) in pUC18	This work
pTPE18-3	*pehA* (N371I) in pUC18	This work
pTPE19-1	*pehA* (K370T) in pUC18	This work
pTPE19-2	*pehA* (K370R) in pUC18	This work
pTPE19-3	*pehA* (K370M) in pUC18	This work



Plasmid	Mutated residues	370	371	372	373	374	375	376 aa
pTPE1	wt	K	N	V	N	V	K	K
pTPE7	V374A	.	.	.	.	A	.	.
pTPE8	V374D	.	.	.	.	D	.	.
pTPE13	N373Y	.	.	.	Y	.	.	.
pTPE14	N373Y-V374A	.	.	.	Y	A	.	.
pTPE15	N373Y-V374D	.	.	.	Y	D	.	.
pTPE16-1	V372A	.	.	A	.	.	.	.
pTPE16-2	V372D	.	.	D	.	.	.	.
pTPE16-3	V372G	.	.	G	.	.	.	.
pTPE17-1	V374L	.	.	.	.	L	.	.
pTPE17-2	V374I	.	.	.	.	I	.	.
pTPE17-3	V374F	.	.	.	.	F	.	.
pTPE18-1	N371S	.	S	.	.	.	.	.
pTPE18-2	N371T	.	T	.	.	.	.	.
pTPE18-3	N371I	.	I	.	.	.	.	.
pTPE19-1	K370T	T	.	.	.	.	.	.
pTPE19-2	K370R	R	.	.	.	.	.	.
pTPE19-3	K370M	M	.	.	.	.	.	.

Fig. 1. C-terminal mutations of PehA. The substituted residue and its location are shown for the seven last amino acids. sp, signal peptide.

substituted for Val-374. In all cases, secretion and stability of the protein were judged unchanged in immunoblot analysis (Fig. 2B), and the level and distribution of Peh activity were very similar to those of the wild-type enzyme (Table 2).

Next, the analysis was extended to position 372. Again, mutants having a V372A or V372D substitution exhibited altered phenotypes, with Asp causing more severe effects than Ala (Fig. 2A). In fact, in immunoblots, the V372A and V372D substitutions closely resembled the respective substitutions of V374 (Fig. 2A). This suggests that probably both positions must be occupied by a hydrophobic amino acid. In accordance with this, even the substitution V372G was expected to affect protein stability, as occurred when Gly was substituted for V374 [20]. However, the V372G mutant appeared stable in immunoblot analysis (Fig. 2A) and showed considerable polygalacturonase activity (Table 2). Some of the mutant protein could also be detected in the culture supernatant, yet the level of extracellular V372G did not exceed that of the unspecific leakage observed for the non-secreting control β-lactamase (Table 2). Further, the mutant protein was localized by osmotic shock treatment to the periplasmic fraction (data not shown).

We also studied the significance of two non-hydrophobic residues, K370 and N371, which are conserved between several eukaryotic and prokaryotic polygalacturonases [20]. Ser, Thr and Ile were substituted for the N371, and Thr, Arg and Met for K370 (Fig. 1). More pronounced effects were caused only by the N371T and N371I substitutions, which rendered the PehA entirely unstable (Fig. 3). The different effects caused by the N371S and N371T substitutions are noteworthy, since Ser and Thr are generally regarded as similar in sequence comparisons. Unlike the observed clear requirement for Asn in position 371, no apparent reason was found for the observed conservation of K370. Three mutants K370T, K370R and K370M showed no significant difference from wild-type PehA by immunoblot analysis (Fig. 3) and enzyme assays (Table 3).

4. Discussion

Previously, we showed that an intact C-terminus is essential for correct targeting of the major secreted enzyme PehA in *Ecc*, and that alterations within the C-terminus also affect protein conformation [20]. In this study, it is shown that a terminal hydrophobic cluster is required both structurally and for correct targeting of the protein. The cluster consists of two aa, V372 and V374. Non-hydrophobic aa, especially Asp, caused in either position severe effects on protein stability, suggesting that the protein conformation was affected. In most cases, decreased stability paralleled a lowered level of secretion. For the latter position, the mutant phenotype exhibited by V374D could be suppressed by a second-site substitution N373Y. Further, all hydrophobic aa substituting for V374 retained the full wild-type characteristics of PehA. The effects of hydrophobic substitutions were not specifically tested for the position 372, but the characteristics of V372A and V372D, as compared to V374A and V374D, suggest that

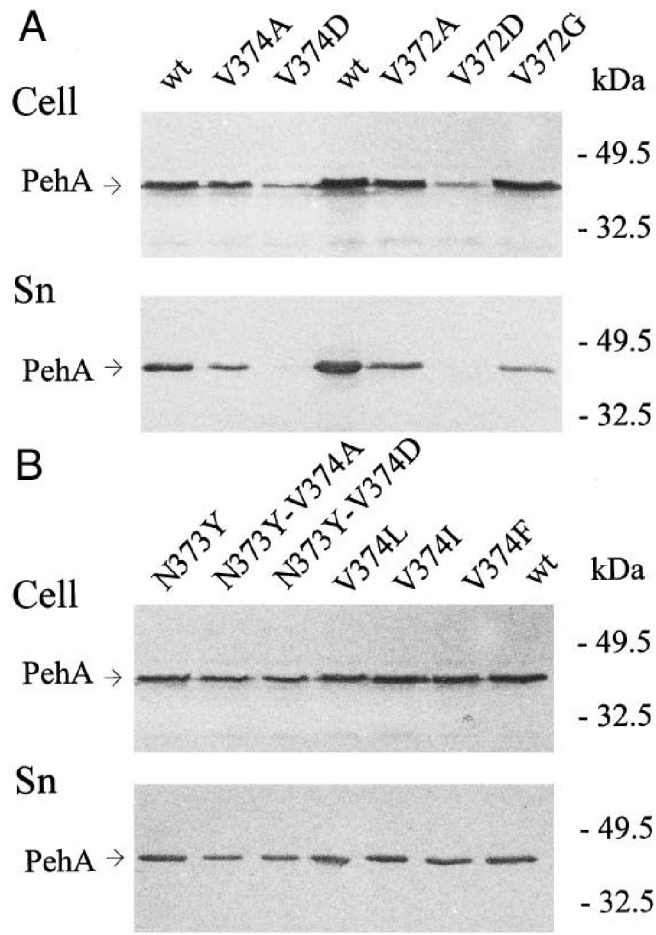


Fig. 2. (A) Analysis of aa substitutions in the positions 372 and 374. HSE2201 cells harboring the various plasmids were grown to a stationary phase in L-medium supplemented with 0.4% glycerol and Ap 150 μg ml^{-1}. 10 μl aliquots of cell and supernatant fractions were subjected to SDS-PAGE and immunoblot analysis as described in Section 2. Cell refers to cellular fraction and Sn to culture supernatant fraction. (B) Effects of hydrophobic substitutions in positions 373 and 374. Analysis was performed as described in panel A.

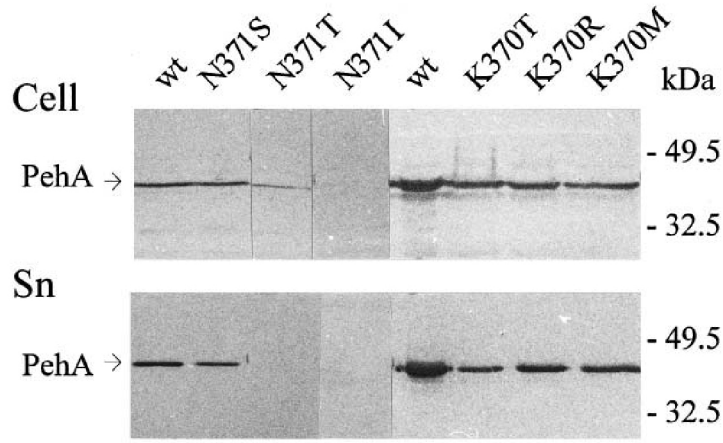


Fig. 3. Analysis of aa substitutions at the conserved residues 370 and 371. Analysis was carried out as described in Fig. 2A.

hydrophobicity, rather than a specific aa, is probably the main criterion for both positions.

As an exception to the rule, one of the analyzed mutants, V372G, was found to be secretion-deficient but still remarkably stable, producing approx. 70% of the cell-bound activity of the native PehA. The mutant protein appeared in the supernatant only in amounts comparable to those of the non-secreting control β-lactamase, suggesting that either the C-terminus of PehA, or residues close to it, could be directly involved in formation of the signal. Although some studies [17,19] depict an N-terminal secretion signal, there is also evidence for a C-terminal targeting information [18], as well as for the existence of a dispersed signal which would occur in distinct domains of the protein [13]. Thus, both the location and composition of the targeting signal recognized by GSPs of different bacterial species might be subject to a marked divergation. Alternatively, even very small changes in the conformation of PehA could affect the mechanism by which it is recognized as secreted. Supposing that formation of an accurately exposed targeting signal would indeed be conformation-sensitive, one might hypothesize, conversely, that the binding of a GSP receptor to the signal could also induce the secreted

Table 2
Effects of mutations in hydrophobic positions 372 and 374 on distribution and levels of Peh activity

Plasmid carried by strain HSE2201	Mutated residue	Peh activity[a] (0.1 μmol min^{-1})			Bla activity[b]
		Cell	Sn	Sn (%)	Sn (%)
pTPE1	wt	8.1	8.3	51	15
pTPE7	V374A	3.3	2.5	43	16
pTPE8	V374D	0.5	0.1	17	14
pTPE13	N373Y	7.3	7.7	51	12
pTPE14	N373Y-V374A	3.9	3.2	45	18
pTPE15	N373Y-V374D	4.9	3.9	44	18
pTPE16-1	V372A	8.3	6.1	42	12
pTPE16-2	V372D	nd	nd	nd	13
pTPE16-3	V372G	5.6	0.6	10	10
pTPE17-1	V374L	8.6	8.1	49	12
pTPE17-2	V374I	8.7	8.4	49	13
pTPE17-3	V374F	8.8	7.9	47	11

[a]Polygalacturonase (PehA) was determined from cell and culture supernatant fractions as described in Section 2 according to Collmer et al. [28]. 1 unit was defined as the amount of enzyme activity corresponding to the release of 0.1 μmol glucose min^{-1} under the conditions used. Sn, culture supernatant. nd, not detectable. The numbers shown represent average values of at least three independent experiments.
[b]β-Lactamase was assayed from cells and culture supernatant as described in Section 2 according to O'Callaghan et al. [29]. Sn (%), percentage of the activity in supernatant fraction of the total activity detected in cell and supernatant fractions.

Table 3
Effects of mutations in conserved positions 370 and 371 on distribution and levels of Peh activity

Plasmid carried by strain HSE2201	Mutated residue	Peh activity[a] (0.1 μmol min^{-1})			Bla activity[a]
		Cell	Sn	Sn (%)	Sn (%)
pTPE1	wt	8.1	8.3	51	15
pTPE18-1	N371S	5.6	3.7	40	10
pTPE18-2	N371T	1.4	nd	nd	12
pTPE18-3	N371I	nd	nd	nd	11
pTPE19-1	K370T	7.9	7.1	47	9
pTPE19-2	K370R	7.4	7.4	50	13
pTPE19-3	K370M	7.3	7.3	50	9

[a]Polygalacturonase and β-lactamase were assayed as in Table 2.

protein to undergo a transient change of conformation during translocation. If correct, this might provide at least a partial explanation for the observations that chimeric proteins, in which the conformation of the passenger molecule most likely cannot be affected by GSP, are often secreted less efficiently through the GSP than the native ones, or not secreted at all [20,30].

In addition to hydrophobic residues, several positions harboring a non-hydrophobic aa were studied. The substitutions N371T and N371I rendered PehA extremely unstable, and little, if any of the mutant proteins could be detected in immunoblots. Thus, the structural importance of N371 is in agreement with its conservation within several polygalacturonases [20], in contrast to the other conserved residue K370, which appeared dispensable. Because the two extreme terminal K375 and K376 are also unimportant [20], all the three terminal lysines seem to lack significance.

Currently, we are performing random mutagenesis over the entire *pehA* to search for stable non-secreting mutants. Identification of regions accumulating mutations that impair the protein with secretion while leaving other properties unchanged should give us tools for the accurate localization and characterization of the targeting signal of the PehA protein.

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