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# Phytotoxic Protein PcF, Purification, Characterization, and cDNA Sequencing of a Novel Hydroxyproline-containing Factor Secreted by the Strawberry Pathogen *Phytophthora cactorum**

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**A novel protein factor, named PcF, has been isolated from the culture filtrate of *Phytophthora cactorum* strain P381 using a highly sensitive leaf necrosis bioassay with tomato seedlings. Isolated PcF protein alone induced leaf necrosis on its host strawberry plant. The primary structure and cDNA sequence of this novel phytotoxic protein was determined, and BLAST searches of Swiss-Prot, EMBL, and GenBank™/EBI data banks showed that PcF shared no significant homology with other known sequences. The 52-residue PcF protein, which contains a 4-hydroxyproline residue along with three S–S bridges, exhibits a high content of acidic sidechains, accounting for its isoelectric point of 4.4. The molecular mass of isolated PcF is 5,622 ± 0.5 Da as determined by mass spectrometry and matches that calculated from the deduced amino acid sequence with cDNA sequencing. The cDNA sequence indicates that PcF is first produced as a larger precursor, comprising an additional N-terminal, 21-residue secretory signal peptide. Maturation of this protein involves the hydroxylation of proline 49, a feature that is unique among other known secreted fungal phytopathogenic proteins.**

In modern agriculture, the selection of pathogen-resistant cultivars remains of the utmost importance. Conventional breeding selection protocols are relatively inefficient as a consequence of the general lack of genetic variability in cultivated plants as well as the inability to keep pace with the rapid adaptation of pathogen genotypes. New strategies, aimed at achieving resistant plants through gene engineering, require an in depth knowledge of the mechanism of pathogenesis at the molecular level. Host-pathogen interactions can result in either "incompatibility" (resistance) or "susceptibility" (pathogenesis). In either case, mounting evidence suggests that the process is mediated by the production of so-called elicitor- and toxin-signaling molecules (1–3). Signal recognition at the plant cell surface triggers an ordered cascade of downstream events, leading to a range of host-cell responses (4–7). Because elicitor and toxic mediators play a central role, elucidation of the mechanism of plant-pathogen interactions promises to provide insights about strategies for incorporating pathogen resistance in cultivated plants.

The European cultivated strawberry plants (*Fragaria vesca* × *ananassa* Duch.) are mostly susceptible to attack by *Phytophthora cactorum* (8, 9), a pathogenic oomycete for many herbaceous and woody plants because of its wide range host specificity. This pathogen is the causal agent of the "leather rot" and "root rot" diseases in strawberry plants, whose morphological symptoms are recognized by rotting of root, crown, and fruit tissues (10). Plich and Rudnicki (11) first reported that culture filtrates of *P. cactorum* possessed phytotoxins with action on tomato and involvement in the development of apple tree diseases. Most of these metabolites, however, have not been purified and characterized. The exception is "cactorein" from *P. cactorum*, a secreted protein that elicits an incompatibility reaction when applied to non-host tobacco, that is reportedly a classical avirulence gene product (12, 13). This elicitor belongs to the 10-kDa elicitin family, a highly conserved protein group from *Phytophthora* spp. showing structural and functional similarities (14, 15).

We recently identified a small cysteine-rich protein endowed with toxic activity on both strawberry and tomato plants that is present in culture filtrates of *P. cactorum* (P381 strain) previously isolated from infected strawberry plants (16). The molecular characteristics of this protein do not possess the properties of known members of elicitins from *Phytophthora* spp. This phytotoxic protein also appears to be different from other agents involved in plant-pathogen interaction. In this report, we describe the isolation, characterization, and molecular cloning of this novel phytotoxic protein, hereby named PcF.[1]

## EXPERIMENTAL PROCEDURES

*Materials*—All chemicals were of analytical grade and were obtained from Sigma, except where indicated. HPLC and FPLC chromatographic procedures were performed on an AKTA Purifier system (Amersham Pharmacia Biotech).

*Fungal Culture—P. cactorum* strain P381, isolated from infected

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This work is dedicated to the memory of Professor Pasquale Rosati.

*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF354650.*

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[1] The abbreviations used are: PcF, derived from the *P. cactorum-Fragaria* interaction system; Pam-PcF, β-propionamidated PcF protein; Pam-Cys, β-propionamidated cysteine; PTH, phenylthiohydantoin; HPLC, high pressure liquid chromatography; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; ISMS, ion spray mass spectrometry; PITC, phenylisothiocyanate; PTC, phenylthiocarbamyl; bp, base pair; DEPC, diethyl pyrocarbonate; RACE, rapid amplification of complementary DNA ends; PCR, polymerase chain reaction; Hyp, hydroxyproline; DTT, dithiothreitol.

strawberry plants, was kindly provided by Prof. G. Cristinzio (University of Naples "Federico II", Italy) to Prof. P. Rosati. They were routinely grown on a solid medium (13.5 g/liter Difco Bacto-agar, 250 ml/liter tomato juice, and 2.7 g/liter $CaCO_3$, pH 6.25) in the dark at 25 °C. Maintenance subculture was carried out every 10–15 days. For growth in liquid culture, a medium deprived of yeast extract and peptone, adapted after Hall *et al.* (17) was used. Growth was carried out in a rotary shaker over 20 days at 25 °C in the dark. Other *Phytophthora* species used, namely *Phytophthora nicotianae*, *Phytophthora cinnamomi*, *Phytophthora cryptogea*, *Phytophthora capsici*, *Phytophthora citrophthora*, and *Phytophthora infestans* were kindly provided by Prof. A. Scala (University of Florence, Italy).

*Bioassays*—Phytotoxic activity of either crude culture filtrate or purified fractions, was routinely assayed on tomato seedlings (18). Tomato seedlings (*Lycopersicon esculentum* cv. Marmande) were grown in a moisture chamber until they had produced only the two cotyledon leaflets. They were then resected near the roots and incubated for 24–36 h in Eppendorf tubes containing 100 μl of the solution to be assayed. Each assay was made in duplicate and controls were performed to test the effect of the various buffers on the viability of the seedlings. The activity was evaluated by the ability of inducing distal necrosis on tomato leaves, and it was scored using an arbitrary scale, ranging from 0 (symptomless) to 5 (complete leaf necrosis and wilting). When appropriate, the toxic activity was assayed directly on *Fragaria vesca* × *ananassa* host plant leaves. The leaves were detached from a mature plant of a strawberry cultivar susceptible to the P381 isolate of *P. cactorum*. 100-μl aliquots of each solution to be assayed were appropriately infiltrated into the lower leaf surface and incubated at room temperature in a moisture chamber. The formation of a necrotic area, localized overleaf to the infiltration site, was then evaluated at intervals after infiltration. Appropriate controls were carried out in parallel to test for the effect of the solvent buffers.

*PcF Protein Purification*—PcF protein was purified starting from 2 liters of *P. cactorum* culture. Mycelia and spores were removed from the exhausted medium by filtration through a 3MM paper (Whatman), followed by centrifugation at 13,000 × *g* for 30 min at 4 °C. The 13,000 × *g* supernatant is referred as the culture filtrate. All the subsequent purification steps were carried out at room temperature.

*DEAE-Sepharose Fast Flow Chromatography*—The *P. cactorum* culture filtrate was adjusted to pH 5.5 with 1 M $NH_4OH$, and loaded onto a DEAE-Sepharose Fast Flow column (2.6 × 9 cm, Amersham Pharmacia Biotech) previously equilibrated with 10 mM ammonium acetate, pH 5.5 (buffer A). After washing the column with buffer A, the elution was carried out isocratically with buffer A containing 1 M KCl (buffer B). A 5 ml/min flow rate was maintained. Aliquots of the eluted fractions were assayed for toxicity on tomato seedlings as described above. Active fractions were combined and added to trifluoroacetic acid and acetonitrile to 0.1 and 7.5% final concentrations, respectively (DEAE-Sepharose pool).

*Resource RPC FPLC Chromatography*—The DEAE-Sepharose pool was filtered through a 0.22-μm membrane (Millipore) and loaded onto a FPLC Resource RPC column (3 ml, Amersham Pharmacia Biotech), previously equilibrated with 0.1% trifluoroacetic acid, 7.5% acetonitrile. The column was eluted at a flow rate of 2.5 ml/min with a discontinuous gradient of acetonitrile obtained with buffers A (0.1% trifluoroacetic acid) and B (0.1% trifluoroacetic acid, 65% acetonitrile). The gradient conditions were: 11.5% buffer B for 16 min; 11.5–60% B in 112 min; 60–100% B in 16 min, and then hold at 100% B for 24 min. Because of the interference of both trifluoroacetic acid and acetonitrile on the tomato bioassay, 100-μl aliquots of each fraction were evaporated under vacuum (Speed-Vac, Savant) and resuspended in distilled water before performing the bioassay. Active fractions were pooled and added to 10 mM ammonium acetate, pH 5.5, 20 mM NaCl. The pH was adjusted to 5.5 with 1 M $NH_4OH$ (Resource RPC pool).

*TSK-DEAE FPLC Chromatography*—The Resource RPC pool was loaded onto a FPLC Spherogel TSK-DEAE column (4 × 300 mm, Altex), previously equilibrated with 10 mM ammonium acetate buffer, pH 5.5, 20 mM NaCl. The elution of the column was carried out with a discontinuous gradient of NaCl obtained with buffers A (10 mM ammonium acetate buffer, pH 5.5, 20 mM NaCl) and B (10 mM ammonium acetate, pH 5.5, 0.5 M NaCl). A flow rate of 1 ml/min was maintained. The gradient conditions were: buffer A from 0 to 5 min; 0–27% buffer B in 50 min; 27–100% B in 5 min, and then hold at 100% B for 5 min.

*LC-18 FPLC Chromatography*—The pooled biologically active fractions from the previous step were directly applied to an FPLC Supelcosil LC-18-DB column (4.6 × 250 mm, Supelco), equilibrated with 0.1% trifluoroacetic acid buffer. The column was eluted at a flow rate of 1.3 ml/min, with a discontinuous gradient of acetonitrile obtained with buffers A (0.1% trifluoroacetic acid) and B (0.1% trifluoroacetic acid, 65% acetonitrile). The gradient conditions were: buffer A from 0 to 10 min; 0–60% buffer B in 80 min; 60–100% B in 4 min, and then hold at 100% B for 15 min. The active pool was dried under vacuum (Speed-Vac, Savant), dissolved in distilled water, and stored at −20 °C.

*Protein Determination*—Protein concentration was routinely evaluated according to Bradford (19), using bovine serum albumin as the standard. The protein concentration of pure PcF protein was determined spectrophotometrically using a molar $\epsilon_{280\,nm}$ of 3,355 $M^{-1}$ $cm^{-1}$, as calculated from the amino acid composition according to Pace *et al.* (20).

*β-Propionamidation*—Pure PcF protein (2 nmol) was subjected to cysteine alkylation with acrylamide, after protein denaturation-reduction in 2% SDS, 0.1 M DTT, according to Brune (21). The resulting β-propionamidated protein (Pam-PcF) was purified by gel filtration on an FPLC Superdex Peptide PE 7.5/300 column (Amersham Pharmacia Biotech), equilibrated and eluted with 0.1% trifluoroacetic acid, 30% acetonitrile.

*Evaluation of Molecular Size*—Relative molecular mass of pure PcF was evaluated by tricine-SDS-PAGE as described by Schägger and Von Jagow (22), with minor modifications. Before loading the gel, the pure PcF protein sample was heated to 100 °C for 5 min in a denaturating mixture containing 2% SDS, 5% β-mercaptoethanol, and 10 mM DTT. Alternatively, precise determinations of the molecular mass of pure native PcF and Pam-PcF proteins were performed by ion spray mass spectrometry (ISMS) on a Navigator apparatus (Finnigan, Manchester, UK).

*Determination of Isoelectric Point*—The isoelectric pH value of PcF was determined by FPLC chromatofocusing. A 5-μg aliquot of pure PcF, dissolved in 25 mM Bis-Tris buffer, pH 6.5, was loaded onto a Mono P HR 5/5 FPLC column (1 ml, Amersham Pharmacia Biotech) previously equilibrated with the same buffer. Elution was performed with 10-fold diluted Polybuffer 74 (Amersham Pharmacia Biotech), pH 4.0.

*Amino Acid Analysis*—4-μg samples of pure PcF protein were heated to 155 °C in evacuated sealed tubes for 90 min in the presence of 6 N HCl. Amino acid analysis was carried out on a Cromakon 500 (Kontron) automated analyzer using an *o*-phthalaldehyde postcolumn derivatization procedure (23). Alternatively, for the determination of the hydroxyproline residues (Hyp), amino acid analysis was performed by HPLC on LC-18 column after a PITC precolumn derivatization, according to Heinrikson and Meredith (24). Cysteine was determined as cysteic acid after performic acid oxidation (25). Tryptophan content was investigated spectrophotometrically, according to Edelhoch (26).

*SH Groups Determination*—Free thiol groups were determined through mass spectrometry analysis by comparison of the native PcF protein molecular mass with the mass of the protein after β-propionamidation in the absence of 0.1 M DTT. Following β-propionamidation, the expected mass increase corresponds to 71 Da per free SH group.

*N-terminal Sequencing*—After spotting the protein (10 nmol) onto a polyvinylidene difluoride membrane, N-terminal sequencing was carried out on both native PcF protein and Pam-PcF protein by automated Edman degradation on a Procise Model 491 gas-phase sequencer, connected with an online phenyl thiohydantoin (PTH) amino acid analyser, model 140C (Applied Biosystems, Foster City, CA). Amino acids were assessed, as their PTH derivatives, on the basis of coelution with appropriate standards (20 PTH-amino acid standard solution, PerkinElmer Life Sciences), with the exception of the β-propionamidated cysteine (Pam-Cys) and the hydroxyproline residues that did not yield any signal matching the above standards. The cysteine residues were identified as PTH-2-propionamide cysteine (21). The 4-hydroxyproline (4-Hyp) residue was identified, as a double peak by comparison with the retention times of expected peaks obtained during the automated sequencing of both [$Hyp^3$]bradykynin (Sigma) and tryptic fragments from human collagen, type IV (Sigma). Both latter proteins are known to contain 4-Hyp at known positions in their primary structure (27, 28). The double-peak yield of PTH-4-Hyp, resulting from the automated sequencing process of the above 4-hydroxyproline-containing proteins, was further investigated, and the two peaks were subsequently identified as *trans*-4-Hyp and *cis*-4-Hyp, respectively, on the basis of coelution with the corresponding genuine standards (Sigma), directly loaded onto the glass fiber disc of the sequencer cartridge, followed by a single Edman cycle.

*Fungal RNA Preparation*—Fresh P381 *P. cactorum* mycelia (100-mg aliquots, wet weight), harvested by filtration from the liquid culture, were collected in DEPC-treated tubes and directly pestle-homogenized in liquid nitrogen. Subsequently, total RNA and poly($A^+$) RNA extractions were carried out with the High Pure RNA Isolation kit (Roche

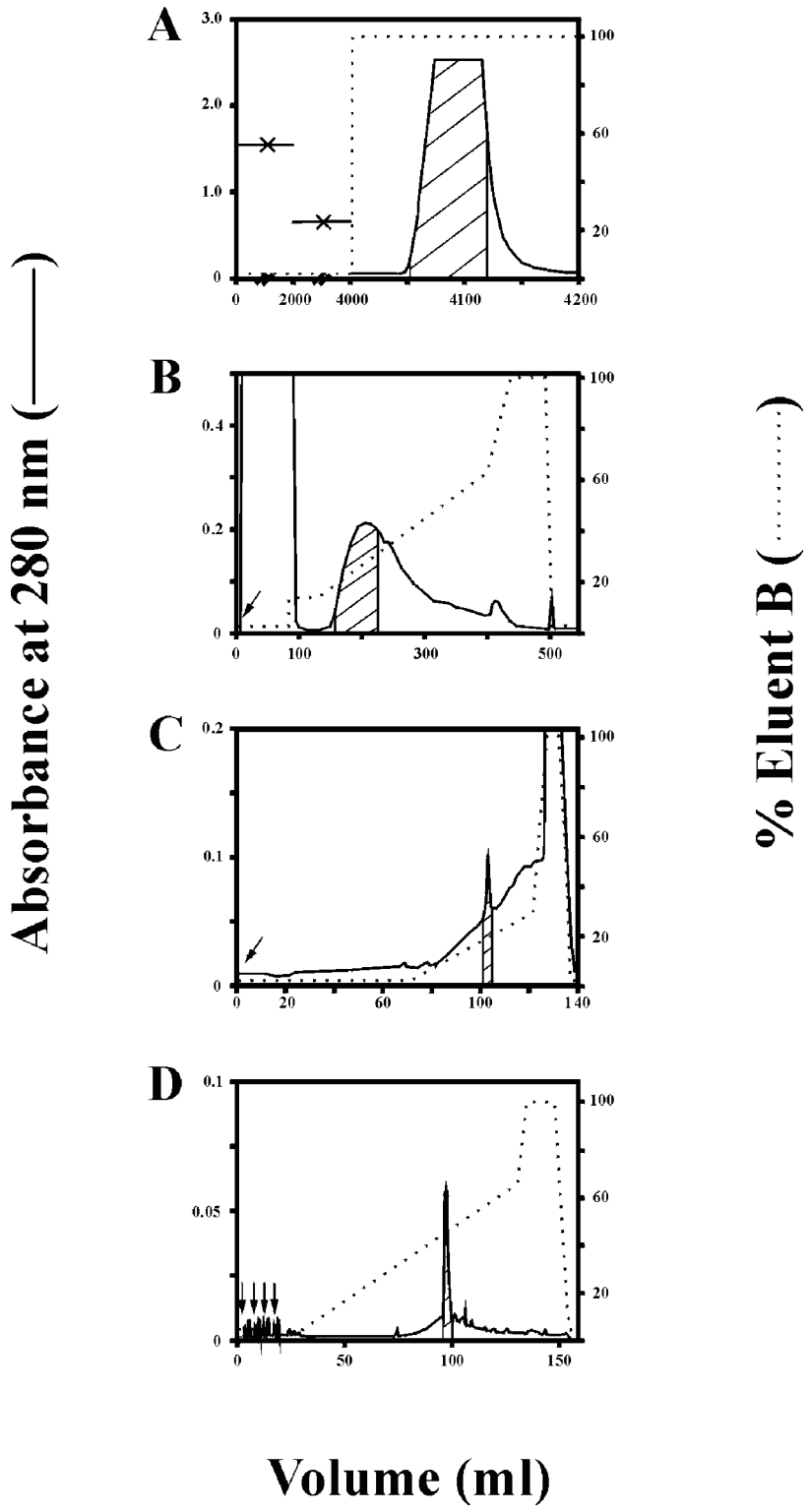


FIG. 1. **PcF protein purification procedure from the culture filtrate of *P. cactorum* strain P381.** The *dotted lines* in the four chromatography profiles indicate the gradient elution conditions (see text for the corresponding buffers), whereas the *hatched areas* indicate the toxic eluted fractions able to induce distal leaf necrosis on tomato seedlings. *A,* DEAE-Sepharose Fast Flow chromatography of the culture filtrate. *B,* Resource RPC FPLC chromatography of the pooled biologically active fractions eluted from the DEAE-Sepharose column. *C,* TSK-DEAE FPLC chromatography of the pooled biologically active fractions eluted from the Resource RPC column. *D,* LC-18 FPLC chromatography of the pooled biologically active fractions eluted from the TSK-DEAE column. The samples were loaded onto the HPLC columns where indicated by *arrows*, either by HPLC pump (*B* and *C*) or by a 1-ml loop HPLC injection valve (*D*).

Molecular Biochemicals) and the mRNA Capture kit (Roche Molecular Biochemicals), respectively.

*RACE Experiments*—A cDNA partial sequence encoding for the PcF protein was obtained from the fungal poly($A^+$) RNA after a one-step cDNA synthesis and amplification (RT-PCR). RT-PCR was directly performed in the oligo(dT)-coated PCR tubes, saturated with poly($A^+$) RNA by the Titan™ One Tube RT-PCR System kit (Roche Molecular Biochemicals). The degenerate oligonucleotide primers used, PcF 1 and PcF 2, are represented in Fig. 3. The RT-PCR conditions were: 30 min at 50 °C; 2 min at 94 °C; 10 cycles (30 s at 94 °C; 30 s at 45 °C; 1 min at 68 °C); 25 cycles (30 s at 94 °C; 30 s at 45 °C; 1 min at 68 °C, with 5-s elongation increase at each cycle); 7 min at 68 °C. Furthermore, both 3′- and 5′-end extensions of the cDNA partial sequence were performed with the 5′/3′-RACE kit (Roche Molecular Biochemicals), according to the instructions and reagents provided by the manufacturer (29). The high fidelity DyNAzyme™ EXT DNA polymerase was obtained from Finnzymes (Finland). The oligonucleotide primers used, designed from the cDNA partial sequence, were as depicted in Fig. 3. The oligo(dT) anchor primer, $V_1$, and the anchor primer, $V_2$, were part of the kit. cDNA synthesis was performed by AMV reverse transcription from 2 $\mu$g of total RNA in the presence of either the PcF 3 primer, for 5′-end extension, or the $V_1$ primer, for 3′-end extension. Thereafter, for 5′-RACE, the single strand cDNA was purified (High Pure™ PCR Products Purification kit, Roche Molecular Biochemicals), and poly(A) tailed at the 3′-end by terminal dTransferase. Tailed cDNA was then used as the template for PCR in the presence of the $V_1$ and PcF 3 primers. A

nested PCR was carried out by diluting an aliquot of the sample in a second PCR mixture containing the $V_2$ and PcF 5 primers. A second nested PCR was further performed, in the same manner with the $V_2$ and PcF 7 primers. Similarly, for 3′-RACE, the single strand cDNA was utilized as the template in the subsequent PCR and two nested PCRs. The anchor primer $V_2$ was used in all amplifications, either together with the PcF 4 primer (first amplification) or with the PcF 6 primer (nested PCR) or with the PcF 8 primer (second nested PCR). Throughout, PCR conditions were as follows: 2 min at 94 °C; 10 cycles (15 s at 94 °C; 30 s at 65 °C; 40 s at 72 °C); 25 cycles (15 s at 94 °C; 30 s at 65 °C; 40 s at 68 °C, with 20-s elongation increase at each cycle); 7 min at 72 °C.

*Cloning and Sequencing*—The cDNA fragments obtained from RACE experiments were re-amplified by PCR in 50-$\mu$l reaction mixtures containing 10 mM Tris-HCl buffer, pH 8.3, 50 mM KCl, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 1 unit of AmpliTaq DNA polymerase (Perkin Elmer), 10 pmol of each primer, and an appropriate amount of the template. PCR conditions were as follows: 96 °C for 7 min; 30 cycles (30 s at 96 °C; 30 s at the appropriate annealing temperature, either 45 °C or 65 °C; 30 s at 72 °C); 72 °C for 30 min. The annealing temperature was either 45 °C, for re-amplification of the first cDNA fragment, or 65 °C, for re-amplifications of both 3′- and 5′-end extension fragments. The amplified cDNA fragments were then purified from the agarose gel and ligated by a standard TA cloning procedure, in the pGEM-5Zf(+) vector (2× Rapid Ligation Buffer kit, Promega). After transformation of the *Escherichia coli* JM109 strain, the recombinant plasmid clones were purified and subjected to automated dideoxy chain-termination sequencing (30).

## RESULTS AND DISCUSSION

*Purification of the PcF Protein*—The presence of a phytotoxic activity in the culture filtrates of *P. cactorum* P381 strain, was determined through bioassays on tomato seedlings (31). We found that about 3 $\mu$g of culture filtrate protein was the minimum required to attain a toxicity score of 5 within 17 h. During subsequent purification, toxic activity was only observed in those chromatographic fractions that yielded the pure PcF protein (Fig. 1). Because there were no other active fractions, the tomato bioassay appears to be highly sensitive and specific for PcF. Furthermore, although the activity resisted to heating at 100 °C for 5 min (32), the proteinaceous nature of the secreted *P. cactorum* toxin(s) responsible for tomato seedling necrosis was indicated by loss of toxicity upon combined Pronase and heat treatments (32).

The protein factor, named PcF, with reference to the organisms *P. cactorum* and *Fragaria* that are involved in the interaction, was purified to homogeneity. The four chromatographic treatments comprising a typical purification procedure are illustrated in Fig. 1. From an initial 2 liters of culture filtrate containing about 50 mg of total protein, the procedure yielded about 200 $\mu$g of pure PcF protein, corresponding to an enrichment factor of 250. The active final preparation exhibited a single protein band on SDS-PAGE (not shown). The absence of protein contaminants was also evidenced by automated Edman degradation sequencing, which resulted in one single N-terminal sequence (see below). The final preparation was used for the subsequent characterization of the PcF protein.

*Biological Activity*—We found that 3–5 $\mu$g of pure PcF protein were sufficient to induce a complete leaf necrosis on the tomato seedling bioassay within 20 h. Under the same bioassay conditions, similar amounts of the $\beta$-propionamidated PcF protein (Pam-PcF) were completely inactive. To evaluate the activity on its strawberry host, purified PcF was also assayed for its ability to induce localized leaf necrosis on a *Fragaria vesca* × *ananassa* cultivar that is susceptible to the *P. cactorum* P381 strain. As shown in Fig. 2, the purified PcF (80 $\mu$g) induced the formation of a marked necrotic area within 24 h after leaf infiltration. Such a level of activity is comparable with that of previously reported phytotoxic fungal proteins toward their hosts (33, 34). Our findings indicate an involvement of the PcF protein in this *P. cactorum-Fragaria* interaction as an host-specific factor. The mode of action of PcF appeared to be consistent with an hypersensitivity response (HR), a distinctive behavior caused by fungal pathogens and their phytotoxic factors (4–7). However, further experimental evidence appears to be needed to establish the specific virulence or avirulence role of PcF protein (*e.g.* either in the *P. cactorum*-strawberry incompatible interaction or in its interaction with other plants).

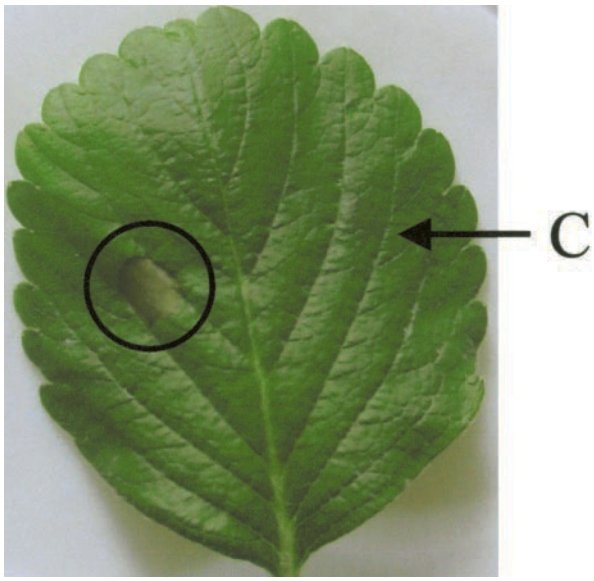


FIG. 2. **Leaf necrosis induced by PcF on host strawberry plant.** Infiltration with 80 $\mu$g of pure PcF protein, dissolved in distilled water, induces localized necrosis on host strawberry (*Fragaria vesca* × *ananassa*) plant leaf (*circle*). Control solution was distilled water (*C*, *arrow*). The necrotic effect was evaluated and recorded at 24 h after infiltration.

By applying the very same PcF purification scheme on the culture filtrates of two other *P. cactorum* strains isolated independently from infected strawberry plants harvested from different Italian fields, we obtained a protein that had six N-terminal residues that were identical to PcF (not shown). In addition, with the specific bioassay on tomato seedlings, the toxic activity was only found in the culture filtrates from *P. cactorum* and *P. nicotianae*; none was found from *P. cinnamomi*, *P. cryptogea*, *P. capsici*, *P. citrophthora*, or *P. infestans*. Furthermore, *P. nicotianae*, a well known tomato pathogen, from which other phytotoxic proteins have been characterized (18, 35), lacked the PcF protein based on the PcF purification protocol. Therefore, the observed constitutive PcF protein expression and secretion from *P. cactorum* RACE, but not from other *Phytophthora* spp., leads us to hypothesize that PcF might be either unique for the *P. cactorum*, or, possibly, for those *P. cactorum* strains that are pathogenic to strawberry.

*Molecular Characterization*—Chromatofocusing of pure PcF protein revealed an acidic isoelectric point of 4.4 ± 0.2. ISMS analysis of the native protein showed a molecular mass of 5,622 ± 0.5 Da. Following $\beta$-propionamidation of the denatured PcF protein in the absence of reducing agents, the same molecular mass of 5,622 Da was obtained, a finding that supports the absence of free thiol groups in the protein. Furthermore, ISMS analysis of Pam-PcF protein, obtained after $\beta$-propionamidation under strongly reducing conditions, revealed a molecular mass of 6,054 ± 0.35 Da, consistent with the presence of 6 oxidized cysteine residues per mol of protein, putatively involved in 3 disulfide bridges. The presence of 6 cysteine residues was confirmed by amino acid analysis of the pure protein. Amino acid analysis also revealed the absence of methionine residues and a relative excess of both hydrophobic and acidic residues, consistent with the observed protein behavior in solution. In addition, spectrophotometric determinations re-

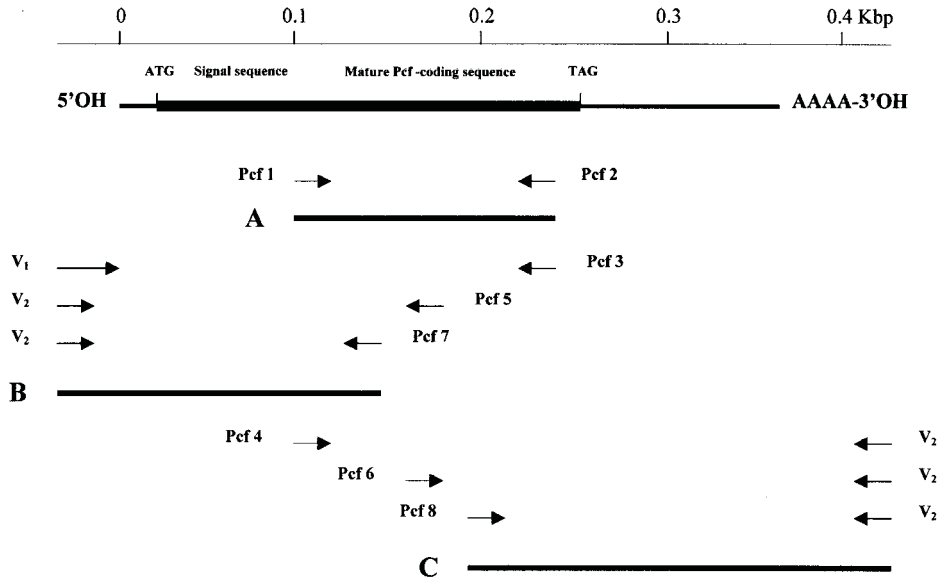


FIG. 3. **Isolation scheme for PcF-coding cDNA.** *Upper*, cDNA encoding for the PcF protein, which has been obtained by overlapping the nucleotide fragments A, B, and C, identified after RACE experiments (see details in the text). *A,* the 144-bp RT-PCR fragment isolated from poly($A^+$) RNA with the PcF 1 and PcF 2 degenerate primers. *B,* 5′-RACE fragment of 179 bp amplified with the PcF 3, PcF 5, and PcF 7 nested primers. *C,* 3′-RACE fragment of 220 bp amplified with the PcF 4, PcF 6, and PcF 8 nested primers. The oligonucleotide primers utilized were: PcF 1, 5′-GARGAYCCICTITACTGYCARGC-3′; PcF 2, 5′-GTIGTIGAICCRCAYTGYTCYTC-3′; PcF 3, 5′-GGTGGTGGAGCCACACTGCT-3′; PcF 4, 5′-GAGGATCCGCTGTACTGTCA-3′; PcF 5, 5′-CCTGGTCACGGCACTCTTTGC-3′; PcF 6, 5′-AGCAAAGAGTGCCGTGACCAGG-3′; PcF 7, 5′-CGGCAAGGTTAGCCTCAGA-3′; PcF 8, 5′-TGGGCGATGATTTCCACAGG-3′. $V_1$ and $V_2$ primers were the oligo(dT) anchor primer and the anchor primer, respectively, supplied by the 3′/5′ RACE kit (Roche Molecular Biochemicals).

vealed the absence of tryptophan residues. SDS-PAGE of pure PcF protein under standard denaturating-reducing conditions (22) shows one single, 15-kDa band (not shown), which is not consistent with the mass value of 5,622 Da obtained by ISMS mass spectrometry. However, the 15-kDa SDS-PAGE band was shifted to an estimated 6-kDa band in the presence of 10 mM DTT in the sample buffer (not shown). Because the ISMS mass value of 5,622 Da excludes the possibility of intermolecular disulfide bridges, the 15-kDa band observed on SDS-PAGE under mild reducing conditions, represents an artifact because of suboptimal SDS-protein binding, arising from inefficient reduction of intramolecular disulfide bridges (48).

A partial N-terminal sequence, corresponding to the first 50 amino acids, was directly determined by automated Edman degradation of the pure PcF protein, demonstrating that the N terminus is unblocked. In addition, automated sequencing did not reveal any heterogeneity, confirming the protein homogeneity. The first residue is represented by a glutamic acid, instead of methionine. The 6 cysteine residues were assigned, as Pam-Cys, at positions 6, 11, 26, 39, 40, and 44. Proline residues were found at positions 3 and 12. The 49th Edman cycle did not yield any signal that would match with any of the standard PTH-derived amino acids, rather showing two unidentified peaks eluting just before PTH-derived histidine and after PTH-derived alanine, respectively. These two peaks were subsequently identified as *trans*-4-Hyp and *cis*-4-Hyp, both on the basis of co-elution with the corresponding genuine standards, and by comparison with the retention times of expected peaks from 4-Hyp-containing polypeptides, as described in the "Experimental Procedures." The presence of *trans*-4-Hyp in the PcF protein native structure was also confirmed by amino acid analysis of the PTC-amino acid derivatives from the hydrolyzed PcF protein, by comparison with *trans*-4-Hyp standard (Sigma). Even though our results do not enable a clarification of whether the *cis*, *trans,* or both isomers are present, these findings appear to be consistent with the presence of a 4-Hyp amino acid residue at position 49 in the mature PcF protein.

The presence of a 4-hydroxyproline residue represents a unique feature of the PcF protein with respect to known fungal phytotoxic proteins (36–41). The involvement of a prolyl 4-hydroxylase (EC 1.14.11.2) in a post-translational hydroxylation of proline 49 is suggested by (*a*) the striking specificity for only one of the three proline residues found in the PcF protein sequence, and (*b*) modification at the C-4 position in the proline ring. Although the function of this post-translational modification of the PcF protein remains to be investigated, several hydroxyproline-rich proteins have been described in the cell wall of pseudo-fungal oomycetes (42). Moreover, 4-hydroxyproline is known to be involved in the-folding and stabilizing of the collagen triple helix (43). This hydroxyimino acid also plays a role in linking arabinose units of carbohydrate moieties attached to the plant cell wall extensins (44).

Even after accounting for the changes in mass resulting from hydroxylation of proline 49 and the formation of three disulfide cross-links, the 50 residues that were sequenced yielded a molecular mass of 5,464 Da, a value that is 158 mass units lower than the ISMS-determined mass of 5,622 Da. This discrepancy suggested that one or two additional residues are present at the C terminus. Because PcF protein lacks methionine residues, thereby precluding CNBr digestion, and because PcF resists digestion by trypsin, Asp-N endoproteinase, and Glu-C endoproteinase (not shown), no additional C-terminal fragments could be sequenced. We also found that the PcF protein is not an inhibitor of any of the above proteases (not shown). To investigate the upstream N-terminal and the downstream C-terminal sequences, we resorted to cDNA cloning and sequencing.

*cDNA Cloning*—Knowledge of the partial N-terminal PcF sequence allowed us to design oligonucleotide primers to identify the PcF coding cDNA by means of RT-PCR. Degenerate primers PcF 1 and PcF 2 correspond to amino acid residues 1–8 and 41–48, respectively (Fig. 3). After RT-PCR, a single 144-bp cDNA was isolated, and the sequence of this cDNA yielded a deduced amino acid sequence that exactly matched amino acid residues 1–48 of the chemically determined protein sequence (Fig. 3*A*). RACE experiments were undertaken to obtain infor-

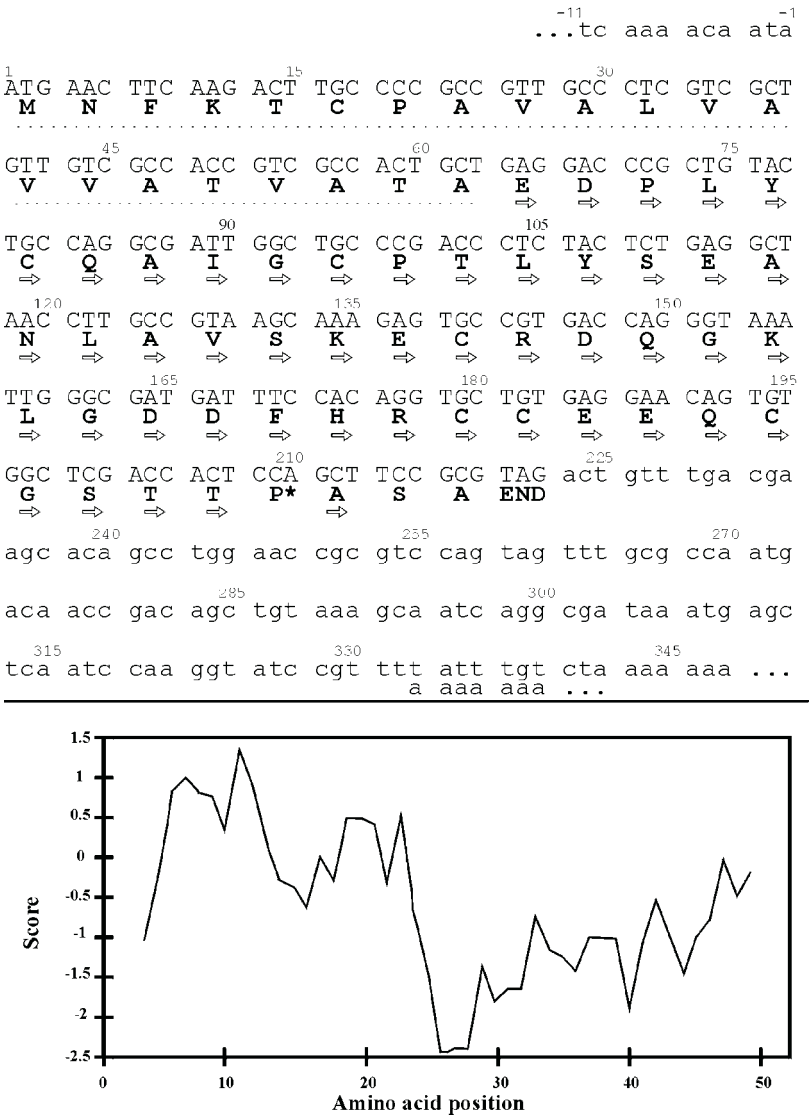


FIG. 4. **PcF-coding cDNA sequence and deduced protein sequence.** *Top,* 254, amino acid sequence obtained by Edman degradation from the whole Pam-PcF protein. *P**, corresponds to the 4-hydroxyproline in the mature protein. The termination codon is marked *END*. The *dotted lines* correspond to a 21-amino acid signal peptide for extracellular secretion. Two different polyadenylation sites of the mRNA are depicted. *Bottom*, the hydropathy profile from the proposed protein sequence was computed with the method of Kyte and Doolittle (46) by the ProtScale program at the Expasy database server (window width of 7). For computing, the 4-hydroxyproline at position 49 was replaced with proline.

mation on both the 3′- and 5′-end of this original fragment, and thus on the complete PcF-coding cDNA sequence. Our hypothesis that PcF protein is likely to be synthesized as a larger precursor is based on the consideration that (i) PcF is a secreted protein, and (ii) that the N-terminal amino acid is a glutamate, as demonstrated by chemical sequencing, and not a methionine. RACE experiments yielded single fragments for both 3′- and 5′-end extensions (Fig. 3, *B* and *C*), as indicated by agarose gel electrophoresis. After cloning and sequencing, we obtained a single 5′-RACE clone of 179 bp and two 3′-RACE clones of 211 bp and 220 bp. Both 3′-RACE clone sequences proved to be identical; however, the larger sequence showed an additional 9-bp sequence at its 3′-end, presumably arising from a different polyadenylation site on the mRNA. All three RACE clones overlapped with the corresponding cDNA ends of the original fragment isolated by RT-PCR, confirming their authenticity. The discrepancies because of the third-base degeneration of the primers originally utilized, were accordingly corrected. Fig. 4 shows the complete cDNA sequence encoding for the PcF protein. A data bank (Swiss-Prot, EMBL, and GenBank™/EBI) survey using the BLAST program failed to yield any significant similarity score with any other known sequences. Furthermore, the PcF protein appears to be unique among other known phytotoxic proteins from *Phytophthora* spp. such as the 10-kDa elicitin family (15, 39) or the 42-kDa glycoprotein elicitor (41).

Upon inspection of the N-terminal region of the PcF-coding cDNA, one finds an ATG start codon 63-bp upstream from the first amino acid in the mature PcF protein. Therefore, the PcF protein appears to be first synthesized as a 73-residue pre-protein precursor, which then undergoes proteolytic removal of a 21-amino acid signal peptide. Indeed, the SignalP program at the Expasy database server predicted that the cleavage site should occur between residues 21 and 22 of the full-length pre-protein sequence, in excellent agreement with experiment. Moreover, the cleaved peptide sequence strikingly conformed to all the proposed criteria for a signal peptide that designates the processed protein for extracellular secretion (45). The C-terminal region was predicted to encode for proline 49, alanine 50, serine 51, and a terminal alanine-52 residue, after which a TAG stop-codon is present. Therefore, the secreted mature PcF protein sequence must comprise 52 amino acid residues. Taking into account the hydroxylation of proline 49 and the occurrence of 3 disulfide bridges, the PeptideMass program at the Expasy server yielded a predicted 5,622.18 molecular mass and a predicted 4.4 isoelectric point, which agree with the experimental data reported above.

The computer-generated hydropathy profile (Fig. 4, *bottom*) indicated the presence of two distinct domains, a predominantly hydrophilic C terminus and a clustered hydrophobic region at the N-terminal. This amphipathic structure has a grand average of hydropathy (GRAVY) score of −0.56, a value

that appears to rule out any ability of the phytotoxic PcF protein to bind to the lipid-rich components of membranes (46). Furthermore, whereas analysis using NetPhos 2.0 strongly suggested serine 44 as a potential phosphorylation site (47), our Edman sequencing gave no indication of any unusual repetitive yield decrease that is characteristic of such modified residues.

In conclusion, we have purified and characterized a novel secreted phytotoxic protein that was assigned the name PcF to indicate the first letters of its organism-of-origin, namely the pathogenic oomycete *P. cactorum*, and its host plant *Fragaria*, the strawberry. Our findings suggest that PcF is most likely a tightly bound structure in its native state, consistent with its observed small size and high, intramolecular S–S bridge content. The phytotoxic PcF protein can be considered to be a new member of the low molecular weight extracellular fungal proteins responsible for pathogenesis in certain host plants (3).

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