ERWINIA CAROTOVORA EXTRACELLULAR PROTEASES: CHARACTERIZATION AND ROLE IN SOFT ROT

by

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ABSTRACT

Erwinia carotovora subsp. carotovora (Ecc) strain EC14, a Gram-negative bacterium, causes soft rot on several crops, including potato. Maceration of potato tuber tissue is caused by secreted pectolytic enzymes. Other cell-degrading enzymes may also have roles in pathogenesis, including cellulases, phospholipases, and protease(s). The objectives of this research were to (1) characterize Ecc extracellular protease (Prt) and (2) elucidate its role in potato soft rot. A gene encoding a Prt, prt1, was cloned from cosmid pCA7 containing Ecc genomic DNA into plasmid pSK1. Escherichia coli transformed with pSK1 or pSK23, a subclone of pSK1, produced intracellularly a 38 kDa Prt with the same pI (4.8) as the secreted Ecc Prt. Prt1 activity produced by E. coli/pSK23 was inhibited by phenanthroline, which inhibits Zn-metalloproteases, but not by Ecc intracellular proteins. Analysis of deletion mutants indicated a 1.2 kb region necessary for Prt1 production. Sequencing of the pSK1 insert revealed a 1,041 bp open reading frame (ORF1) corresponding to the prt1 region. ORF1 encodes a putative polypeptide of 347 amino acids with a total molecular mass of 38.8 kDa. The location of the prt1 promoter was determined to be 173 to 1,173 bp upstream from ORF1 by constructing

transcriptional fusions to lacZ in plasmid pCD267. Primer extension revealed the start of prt1 mRNA 205 bp upstream of ORF1. The deduced amino acid sequence of the prt1 was compared to other proteases; it is similar to several bacterial Zn-metalloproteases. Prt1 production by Ecc was not observed during growth in rich broth; however, Northern analysis showed prt1 mRNA accumulation in Ecc grown in planta. The role of prt1 in soft rot was determined by constructing a Prt1-deficient Ecc; prt1 insertionally inactivated by a kanamycin resistance gene was used to replace wildtype prt1 in the Ecc genome by homologous recombination. This mutant (L-957) had approximately 60 to 80% reduced Prt activity suggesting the presence of a second Prt (Prt2). Prt2 was purified from Ecc culture supernatant. This protease, also a metalloprotease, has a molecular mass of 45 kDa and pI of 4.8. Its amino terminal sequence had significant sequence identity to metalloproteases from Erwinia chrysanthemi and Serratia marcescens, but not to Prt1. Further, unlike Prt1, Prt2 was inhibited by Ecc intracellular proteins. The effect of proteases in potato tuber maceration was measured using L-957 and L-763, a Tn5 transposon mutant constructed previously. L-763 had no extracellular protease activity and may have been mutated in a regulatory region. Both mutants macerated significantly less tuber tissue than the wildtype Ecc. Reduced maceration of L-957 and L-763 was correlated with slower in planta growth. This suggests Prt1 production provides a nutritional advantage for Ecc growth on potato.

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I. LITERATURE REVIEW

SOFT ROT

Introduction

Soft rot is a plant disease that affects many monocotyledonous and dicotyledonous species. In potato (Solanum tuberosum L.), soft rot leads to the maceration of the tuber parenchyma tissue. The disease is caused by soft rot erwinias, clostridia, bacilli, and fluorescent pectolytic pseudomonads. Soft rot due to erwinias has received considerable attention economically since it causes annual losses estimated in 1980 to be greater than $100 million worldwide in the field and storage (Pérombelon and Kelman, 1980). Among the erwinias, the most important soft rotting species are Erwinia carotovora subsp. carotovora (Jones) Bergey et al. (Ecc), Erwinia carotovora subsp. atroseptica van Hall (Eca), and Erwinia chrysanthemi Burkholder et al. (Ech). These are Gram-negative, non-spore-forming facultative anaerobes that belong to the family Enterobacteriaceae. The geographical distribution of various erwinias reflects their optimal growth temperatures. Eca (26°C optimum) is mainly found in cool temperate climates, while Ech (34 to 37°C optima) is responsible for soft rot in tropical and subtropical areas. The optimal growth temperatures of Ecc (28 to 30°C) overlaps with cool and tropical climates, and thus, it has the widest geographical distribution. Ecc and Eca do not seem to have any host-specificity, while Ech has some host specificity (Dickey, 1979). Environmental conditions are important for pathogenesis. High temperatures and humidity and low oxygen concentrations increase disease severity.

LITERATURE REVIEW

Soft rot caused by Ecc

Ecc can be found in plant debris and the rhizosphere and phylloplane of several apparently healthy plants. Evidently, plants may provide nutrients for growth, although bacterial growth does not always lead to disease (reviewed by Stanghellini, 1982). Like many other phytopathogenic bacteria, Ecc survives poorly in the soil in the absence of living hosts. Even on hosts in which Ecc causes disease, it usually exists as a harmless epiphyte. Disease occurs when the resident phase of Ecc switches into a pathogenic phase probably due to changes in the environment that also reduce the ability of the host to defend itself. Ecc usually enters the host through wounds or natural openings such as hydathodes, lenticels, lateral root openings, or stomata, since it is unable to penetrate intact plant surfaces (Huang, 1986). Lenticels are especially important penetration sites in potato tuber soft rot (Adams, 1975). The pathogen then spreads via intercellular spaces among the parenchyma cells. Soft rot begins with water-soaking and discolorization of the plant tissue, followed by reduced turgidity, increased electrolyte leakage, and, eventually, extensive tissue maceration.

Tissue maceration has been attributed to several pectolytic enzymes, but other cell-degrading enzymes secreted by Ecc may also contribute, including cellulases, phospholipases, and proteases (Roberts et al., 1986; Tseng and Mount, 1973). Individual roles for each enzyme involved in causing symptoms are not fully understood. Information obtained from studies involving Ecc and other erwinias has been

extrapolated in trying to draw a picture about the disease mechanism. So far, the pectolytic enzymes have been best characterized and clearly play an important role in soft rot (Collmer et al., 1982; Collmer and Keen, 1986; Kotoujansky, 1987). Pectolytic enzymes include endo-pectate lyase (PL), exo-PL, endo-polygalacturonase (PG), exo-PG, oligo-galacturonide lyase (OGL), pectin lyase, and pectin methylesterase. Several of these enzymes are produced in multiple isoforms with differing isoelectric points, the significance of which is not known. The lyases usually require calcium ions for activity. Pectolytic enzymes are involved in cleaving the α-1,4-bonds between the galacturonan moieties of pectic substances, which are the major components in the middle lamella and primary cell wall of the plant cell. Since the middle lamella and primary cell wall form the first barriers to bacterial penetration, the pectolytic enzymes are, at least, required for the initial steps of soft rot pathogenesis.

The development of molecular techniques allows us to study the individual role for each enzyme. Previously, it was only possible to assess the effect of purified enzymes; now it is feasible to ask the same question by using cloned genes in a different genetic background, such as Escherichia coli (Migula) Castellani and Chalmers, or by mutating a cloned gene followed by homologous recombination with the wildtype gene resulting in a bacterium defective in a particular gene. These techniques have shown that pectolytic enzymes alone are insufficient to cause soft rot. Only limited maceration was caused by E. coli containing cloned Ecc genes for both pectate lyase and

polygalacturonase; no maceration was observed in the presence of either gene alone (Roberts et al., 1986). A constructed mutant of a related pathogen, Ech, inactivated for all of its four pectate lyase genes was still able to cause limited potato tuber maceration (Ried and Collmer, 1988). Therefore, the role of Ecc pectolytic enzymes may be to 'loosen up' the cell wall to allow greater accessibility for the other enzymes secreted by Ecc. Recently, Kelemu and Collmer (1990) found evidence that additional pectate lyase genes, inducible only in planta, occur in E. chrysanthemi.

The function(s) of phospholipase, cellulase, and protease are less well understood. Neither purified protease nor phospholipase can cause tissue maceration alone, but both lyse isolated cucumber (Cucumis sativus L.) protoplasts (Tseng and Mount, 1973). Phospholipase A from a bacteriocinogenic strain of Ecc has been characterized (Itoh et al., 1981). It hydrolyses phosphatidyl glycerol to fatty acids and lysophospholipids and requires calcium for activity. Several Ecc-secreted enzymes require calcium ions, which are abundant in the pectic fraction of the plant cell wall. Ecc is able to grow on cellulose as a sole carbon source and, thus, probably contains all the enzymes needed for cellulose utilization (Mount et al., 1979). The cel genes encoding for cellulolytic activity have been cloned from three strains of Ech (Boyer et al., 1984; van Gijsegem et al., 1985) and from Ecc (Allen et al., 1986; Roberts et al., 1986). One of the cellulolytic enzymes, endo-glucanase, has been purified from Ech. It has a molecular mass of 43 kDa, a pI of 4.3, and is active between pH 6.2 and 7.5 (Boyer et al,

1984). The role of cellulases in maceration has not been assessed. Ecc extracellular protease(s), the subject of my dissertation, is discussed later in this chapter.

The preceding discussion indicates that several genes already identified in Ecc are important for successful pathogenesis. However, many factors may enhance virulence, which, in this dissertation, will mean factors affecting the severity of disease symptoms. These may include protein transport, signal transduction, and pathogen fitness, many of which have not been characterized. Clearly, in the case of Ecc, pathogenicity is based on several factors. Studies of PL and its synthesis during soft rot have also shown that enzyme regulation, rather than its mere production, is critical in pathogenesis.

Regulation of soft rot

Disease based on multiple factors requires regulation of each factor in order to establish an interaction between two organisms and to avoid wasteful synthesis under unfavorable conditions (Lamb et al., 1989; Miller et al., 1989). To initiate the interaction, a pathogen must recognize the presence of a susceptible host and/or environmental conditions (anaerobiosis, temperature, pH, etc.) favorable for disease development. Correct signals lead to induction of genes involved in pathogenesis. This often means rapid activation of several unlinked genes suggesting induction by a common regulatory protein (Kotoujansky, 1987; Miller et al., 1989). Alternatively, sequential induction of genes involved in pathogenesis caused by sequential appearance of degradation products may be another way to regulate virulence (Cooper,

1983; Yang, 1990).

Global gene regulation in a plant-bacterial interaction has received little attention with the notable exceptions of Agrobacterium spp. and Rhizobium spp. (Downie and Johnston, 1986; Peters and Verma, 1990). In both rhizobia and agrobacteria, the factors involved in the interaction are subject to coordinate regulation, where the regulatory system responds to environmental signals. Agrobacterium tumefaciens (Smith and Townsend) Conn enters the plant though a wound and transforms susceptible plant cells to induce tumors by transferring a piece of plasmid DNA (T-DNA) into the plant nuclear genome. In agrobacteria, the genes required for the T-DNA transfer are called virulence genes and are activated by phenolic compounds such as acetosyringone produced by wounded plants (Stachel et al., 1985; 1986). Effective acetosyringone induction requires proper physiological conditions, one of which is low pH (Vernade et al., 1988). Sensing correct environmental conditions is based on a two-component system; one component, the virA product, acts as a membrane-bound sensory component and transmits the signal to the regulatory component, the virG product, probably by protein phosphorylation (Downie and Johnston, 1986; Miller et al, 1989; Ronson et al., 1987). The activated regulator alters gene expression by binding to the positive regulatory sequence (vir-box) located upstream of the virulence genes. Similar two-component regulation of virulence genes containing genes for toxin production, thermoregulation, host colonization, and inactivation of host defense proteins has been observed in the bacterial human pathogens Vibrio cholerae Pacini and

Bordatella pertussis (Bergey et al.) Moreno-Lopez (reviewed in Miller et al., 1989). Rhizobia, involved in nitrogen-fixing root nodules in leguminous plants, enter though intact plant surfaces. They recognize host-synthesized flavones, which stimulate nodulation gene (nod) expression in the bacteria (Djordjevic et al., 1987; Peters et al., 1986; Redmond et al., 1986). Optimal concentrations of flavones for induction occur near emerging root hairs, which are the most favorable sites for rhizobia attachment and penetration (Redmond et al., 1986). The signal transduction leading to the induction of nod genes is a one component system, where a regulatory protein, nodD product, belonging to the LysR family, binds to positive regulatory sequences (nod box) upstream of nod operons (Henikoff et al., 1988).

A recent approach to identify "new" genes important to pathogenesis in plant pathogens has been to look for plant-induced genes. These genes have been identified in Xanthomonas campestris pv. campestris (Pammel) Dowson (Xcc) (Osbourn et al., 1987) and Ech (Beaulieu and van Gijsegem, 1990). Plant-induced mutants of Ech were created using Mu-transposons carrying a promotorless neomycin phosphotransferase gene encoding kanamycin resistance (Kan^r) as a selectable marker. Phenotypically, mutants containing plant-inducible genes were Kan^- on minimal plates but Kan^r in the presence of plant extract. Characterization of these mutants is on-going, but most of the induced genes seem to be involved in pathogenesis. In Ech, cellulase, protease, and four of five pectolytic enzymes (PL_b, PL_c, PL_d, and PL_e) were not induced by plant extract; all the mutants were able to degrade

carboxymethyl-cellulose, skim milk, and polygalacturonate, respectively. Two conclusions can be drawn from these results. First, additional, unknown factors are required for disease initiation. Second, if cellulase, pectolytic enzymes, and protease are induced during pathogenesis, some degradation products from the early steps rather than the initial plant extract act as the inducer.

Regulation of soft rot in Ecc has been studied by examining expression of genes known to be involved in pathogenesis, particularly the genes for pectolytic enzymes (reviewed by Collmer et al., 1982; Kotoujansky, 1987). Among the pectolytic enzymes, PL is induced by plant cell wall derivatives, such as polypectate and other polygalacturonic acid polymers; saturated or unsaturated di-galacturonic acids; or ketodeoxygluconates which are released from plant cell walls by basal levels of exo-PG or exo-PL. In Ecc, exo-PL seems to be important for the production of unsaturated digalacturonates that activate further production of pectolytic enzymes (Yang, 1990). During soft rot, mRNA for exo-PL appears first, followed by mRNA peaks for endo-PL and endo-PG (Yang 1990). Similar sequential appearances of enzymes have been observed in plant pathogenic fungi in planta (Cooper, 1983; Jones et al., 1972). This suggests that specific enzymes are induced by different signal molecules as plant cell walls are progressively degraded.

Regulation by cAMP may be part of the global regulation of pathogenicity genes. cAMP has the characteristics required to signal environmental change; it is a small molecule that is rapidly synthesized

and degraded. In Salmonella typhimurium (Loeffler) Castellani and Chalmers, cAMP regulates genes involved in pathogenesis and a cAMP-deficient mutant is nonpathogenic (Curtiss and Kelly, 1987). Since a cAMP-deficient mutant of Ecc is reduced in the ability to synthesize PL due to catabolite repression (Mount et al., 1979; Roberts et al., 1986), it may be expected that its virulence would be reduced. In Ech, pel and cel genes are also subject to catabolite repression (Boyer et al., 1984; Chambost, 1986). However, neither the cAMP-deficient mutants of Ecc nor Ech have been tested for pathogenicity.

For plant pathogens, the ability to grow in tissues of living plants is critical during pathogenesis. During the transition from a nutrient-poor plant surface to the nutrient-rich environment within plants, bacteria modify their enzyme synthesis to accommodate changed concentrations and types of nutrients, to modulate uptake of nutrients, to reroute metabolic pathways for new carbon sources, to avoid possible blockages due to specific nutrient limitation, and, finally, to coordinate synthetic rates to maintain balanced growth. In erwinias, degradation products of pectolytic enzymes are transported into bacteria and used as carbon sources. However, it has been shown that pectate utilization is not necessary for soft rot; an OGL-deficient mutant of Ech, unable to grow on galacturonan or its derivatives, is able to macerate potato tubers (Chatterjee et al., 1985; Collmer et al., 1982; Collmer and Keen, 1986). Intracellular OGL, which cleaves both saturated and unsaturated digalacturonic acid to ketodeoxygluconates (the most efficient inducers of PL), is the first of the three enzymes

in the pathway required for oligogalacturide catabolism. Thus, pectate utilization is not necessary for pathogen nutrition, indicating that erwinias use other substrates for growth.

An effective iron acquisition mechanism based on high-affinity iron-chelating agents, siderophores, is probably an important factor for pathogen growth in planta, an iron-poor environment, where there is competition between the host and pathogen for iron. Ech mutants defective in iron assimilation are reduced in virulence (Expert et al., 1986; Expert and Toussaint, 1985).

PLANT PROTEINS INVOLVED IN CELL WALL STRUCTURE AND DEFENSE

Introduction

A variety of proteinaceous materials, susceptible to proteolysis, exists in plants. Among the putative substrates for Ecc protease(s) are structural and defense-related proteins located in the plant cell wall and several soluble proteins identified in plant defense. Degradation of plant defense and/or structural proteins by a pathogen could affect the ability of the host to defend itself. The following description is intended to help in understanding the impact of the putative destruction of cell wall and defense proteins.

Knowledge of plant cell wall structure is vital to understanding the function of Ecc hydrolytic enzymes. Since this subject has been reviewed in detail (Darvill et al., 1980; McNeil et al., 1984), I will describe it here only briefly. The plant cell wall surrounds the plasmalemma and is divided into three regions; middle lamella, primary

cell wall, and secondary cell wall. The middle lamella, the outermost layer, is composed mainly of pectic substances of α-1,4-linked galacturonan forming a matrix joining adjacent plant cells together into contiguous tissues analogous to the function of collagen in animals. The primary cell wall is more complex and contains pectic substances, cellulose, hemicellulose, and protein. The secondary wall is synthesized last by the mature cells. It often contains distinct layers of cellulose, more hemicellulose than the primary wall, and lignins that provide structural strength and resistance to enzymatic and microbial degradation.

Plant cell wall proteins and their putative functions

Proteins compose 10 to 20% of the primary cell wall (McNeil et al., 1984). Most are glycoproteins associated tightly with the cell wall via covalent bonds, while other proteins can be extracted with salts. Common glycoproteins found in the plant cell wall are hydroxyproline-rich glycoproteins (HRGPs), in which hydroxyproline is the dominant amino acid. At least three classes of HRGPs have been identified: primary cell wall HRGPs, also known as extensins; arabinogalactan proteins localized mainly in the extracellular matrix and sometimes associated with the plasma membrane (Fincher et al., 1983); and certain lectins found in the Solanaceae family (agglutinins) (Lamport and Catt, 1981). Synthesis of these compounds involves several post-translational modifications; proline-rich polypeptides are extensively hydroxylated to form hydroxyproline-rich polypeptides. Before transport to their final location, most of the hydroxyproline

residues (up to 70%) are also glycosylated to stabilize the polyproline helical structure.

Extensins have been isolated from carrot (Daucus carota var sativa [L.] DC) roots (Stuart and Varner, 1980), soybean (Glycine max [L.] Merr.) seed coats (Cassab et al., 1985), and cultured tomato (Lycopersicon esculentum Mill.) cells (Smith et al., 1984). Carrot HRGPs contain 46% hydroxyproline and are composed of one third protein and two thirds carbohydrate (3% galactose and 97% arabinose). Extensin is also rich in serine, valine, tyrosine, and lysine, and is thus a highly basic protein. After secretion to the primary cell wall, extensin is crosslinked by isodityrosyl residues forming an extensin network (McNeil et al., 1984). This network, originally proposed by Lamport (reviewed in Lamport and Catt, 1981), consists of a mesh of extensin that penetrates cellulose microfibrils in primary cell walls and is suspended in a hydrophilic pectin-hemicellulose gel. Although evidence for this model is lacking and difficult to obtain, a structural role for extensin is supported by studies of the algae, Chlamydomonas reinhardtii Dang. Its cell wall is made entirely from HRGPs, which form a framework responsible for the shape and integrity of the cell (Adair and Appel, 1989). The HRGPs of C. reinhardtii resemble higher plant extensin in high hydroxyproline and serine content with a large proportion of residues in a polyproline helical conformation (Homer and Roberts, 1979). The structural role of extensin in higher plants is supported by its insolubility, its animal collagen-like rod structure, and its developmental and tissue specific regulation (Cassab et al.,

1985; Hong et al., 1987; Tierney et al., 1988).

Besides the structural role of extensin, it may also be involved in cellular defense reactions against physical damage and degradation by pathogens. Extensin mRNAs and polypeptides accumulate after wounding (Chen and Varner, 1985) and pathogen challenge (Mazau and Esquerre-Tugaye, 1986; Mazau et al., 1987; Rumeau et al., 1990; Showalter et al., 1985) and has been correlated with expression of disease resistance (Esquerre-Tugaye et al., 1979; Hammerschmidt et al. 1984; Showalter et al., 1985). Thus, increased extensin synthesis may act as a general defense mechanism by forming structural barriers to prevent pathogen spread and/or repairing damaged cell walls.

Some plant organs contain little if any extensin, instead their cell wall proteins are rich in glycine (up to 70%) (Varner and Cassab, 1986). These glycine-rich proteins (GRPs) may replace extensin in structural function in certain cells. They have been identified in pumpkin (Cucurbita pepo L.) seed coat (Varner and Cassab, 1986), auxin-deprived strawberry (Fragaria chiloensis var. ananassa [Duchesne] Bailey) fruits (Reddy and Poovaiah, 1987), petunia (Petunia hybrida Vilm.) leaves, stems, and flowers (Condit and Meagher, 1986), and in young hypocotyls and ovary tissue of bean (Phaseolus vulgaris L.) (Keller et al., 1988). GRP mRNA and polypeptides accumulate in response to development and wounding (Condit and Meagher, 1987; Keller et al., 1988).

Lectins are glycoproteins located in the plant cell wall that are characterized by their ability to bind to specific carbohydrates such as

N-acetyl-D-glucosamine oligomers and act as strong hemagglutinins (Allen and Neuberger, 1973; Etzler, 1985; Lord, 1985). Solanaceous lectins are sometimes called agglutinins and have been isolated from potato tubers and fruits, and from tomato fruits (reviewed in Showalter and Varner, 1989). They are distinguished from other plant lectins by their higher carbohydrate (50%; 90% arabinose and 10% galactose) and hydroxyproline (50 to 60%) contents, thus resembling extensins (Leach et al., 1982a). Although the function of lectins is still a matter for speculation they have been implicated in plant-microbe recognition by binding to bacterial lipopolysaccharides (Barondes, 1981; Callow, 1977). The specific recognition of symbiotic microorganisms, such as rhizobia, by legumes may be based on lectins (Bauer, 1981). Lectins may also contribute to plant defense by agglutinating incompatible races of pathogenic bacteria and fungi (Callow, 1977; Pistole, 1981; Sequeira, 1978); lectins agglutinate certain incompatible races of the bacterial wilt pathogen, Pseudomonas solanacearum (Smith) Smith in potato and tobacco tissues (Leach et al., 1982b; Sequeira and Graham, 1977). They also precipitate bacterial lipopolysaccharides (Graham et al., 1977), probably because of their high positive charge. The type of lectins present in tubers may affect potato tuber resistance to pathogenic fungi. This is suggested by the observation that lectin preparations from potato cultivars differing in susceptibility to Phytophthora infestans (Montagne) de Bary also have different properties (Andreu and Daleo, 1984; 1988). No correlation among incompatible races of pathogen and agglutination has been found in the involvement of potato lectins in

resistance to soft rot erwinias (Ghanekar and Pérombelon, 1980).

Several proteins inhibitory to microbial cell wall-degrading enzymes have been reported in plant cell walls. These include proteins from sweet potato (Ipomoea batatas [L.] Lam.) inhibiting pectolytic enzymes (Uritani and Stahmann, 1961) and polygalacturonase-inhibiting proteins from bean (Cervone et al., 1987), tomato, sycamore (Platanus areifolia [Ait.] Willd.) (Albersheim and Anderson, 1971), and pear (Pyrus communis L.) (Abu-Goukh et al., 1983). Cell walls also contain proteins capable of inhibiting polysaccharide-degrading enzymes secreted by fungi (Jones et al., 1972).

Cell walls contain a variety of hydrolytic enzymes such as peroxidases, glucanases, phosphatases, and cellulases (Lamport and Catt, 1981). Some of them are involved in the synthesis and turnover of plant cell wall components, but those that do not have a known function in the plant cell wall are considered "secondary" proteins. Their potential to disrupt microbial structures and their cell wall location may allow them to act as defenses against pathogens. Peroxidases, for example, have been shown to be induced in wounded potato tubers and tomato fruits (Roberts et al., 1988). The induction of anionic peroxidases is faster and higher in resistant tomato lines compared to near-isogenic susceptible lines when wounded and challenged with fungal elicitors (Mohan and Kolattukudy, 1990). They probably contribute to defense by reinforcing the cell walls and thus limiting pathogen ingress.

Plant soluble proteins involved in defense

Vacuolar proteins have been considered another plant defense

arsenal. Many lytic proteins and preformed products of secondary metabolism are stored in vacuoles. These substances are released upon vacuole disruption when cells collapse or are damaged. This can occur during a hypersensitive reaction caused by incompatible pathogens or by bursting of plant cells caused by bacterial cell-degrading enzymes.

Plant chitinases are located mainly in vacuoles and intercellular spaces. They are probably involved in plant defense, since their substrate, chitin, is present in the cell walls of many fungi, but not in plants. Chitinases have been purified from many plants such as wheat (Triticum aestivum L.) (Molano et al., 1979), tomato (Pegg and Young, 1982), bean (Boller et al., 1983), and soybean (Wadsworth and Zikakis, 1984). Interestingly, chitinases from bean (Boller et al., 1983), Virginia-creeper (Parthenocissus quinquefolia [L.] Planch.) (Bernasconi et al., 1987), and cucumber (Metraux et al., 1989) also possess lysozyme activity. The ability to hydrolyze peptidoglycan of bacterial cell walls is considered an important animal defense mechanism against pathogenic bacteria (Jolles and Jolles, 1984).

Protease inhibitors, stored also in vacuoles (Holländer-Czytko et al., 1985; Walker-Simmons and Ryan, 1977), are concentrated in storage organs such as seeds and tubers (Laskowski and Kato, 1980; Richardson, 1977; Ryan, 1973). Three types of protease inhibitors have been identified in tomato and potato. Protease inhibitors I and II inhibit serine proteases from a wide variety of microorganisms and insects. A third class, carboxypeptidase inhibitors, inhibit animal pancreatic metalloproteases (Hass and Ryan, 1980; Hass et al., 1981). Protease

inhibitors are induced systemically upon wounding or insect attack and accumulate in leaves (Keil et al., 1986; Ryan, 1988; Sanchez-Serrano et al., 1986; Walker-Simmons and Ryan, 1986).

Other putative defense proteins

Bacterial growth is sometimes affected by plant proteins; cereals have been shown to contain purothionins in the endosperm, which inhibit the growth of pathogenic bacteria in vitro (de Caleya et al., 1972). Barley (Hordeum vulgare L.) leaves also contain thionins, which are basic, highly abundant, low molecular weight polypeptides localized both in the cell wall and vacuoles (Bohlmann et al., 1988; Reimann-Philipp et al., 1989). They are highly toxic to microorganisms and plant protoplasts, probably by disrupting cell membrane permeability. Their localization and the fact that their synthesis can be triggered by pathogens suggests that they may be involved in plant defense.

Patatin in potato tubers accounts for 20 to 30% of the total soluble protein and is a lipid acyl hydrolase (Racusen, 1984). This enzyme may have a secondary function in defense by degrading membranes of pathogens. It releases a free fatty acid, arachidonic acid, from P. infestans mycelia (Bostock et al., 1981), which stimulates phytoalexin synthesis in potato tuber discs. Pretreatment with arachidonic acid renders potato tubers resistant to Ecc (Yang, 1990).

A class of defense proteins with no known enzyme activity is called "pathogenesis-related proteins" (PR-proteins) (Hedrick et al., 1988; Somssich et al., 1986) and/or "wound-induced proteins" (Stanford et al., 1990). They represent mostly low molecular weight, protease-

resistant proteins that are extractable at low pH (Pierpoint et al., 1981). These proteins are induced rapidly upon wounding and accumulate predominantly in the intercellular spaces (Carr et al., 1987). Some PR-proteins have now been identified as chitinases (Legrand et al., 1987) and ß-glucanases (Kauffmann et al., 1987).

BACTERIAL PROTEASES

Introduction

Proteases are widespread in living organisms. Several have been identified in bacteria, although their specific physiological roles are often not known. Most intracellular proteases are probably involved in processing and degradation of proteins and, thus, serve regulatory and nutritional functions (Bond and Butler, 1987; Lazdunski, 1989). Roles for extracellular proteases in bacteria have been less well characterized. However, in most cases studied, proteases secreted by Gram-negative bacteria have been involved in pathogenesis (Hirst and Welch, 1988); the advantage of secreted toxins and degradative enzymes is that they can act on targets at a distance from the pathogen.

Classification of proteolytic enzymes

Proteases are enzymes that hydrolyze peptide bonds (classified by the Enzyme Commission of the International Union of Biochemistry as EC 3.4). They are divided into proteinases and peptidases (Barrett, 1986). Peptidases digest polypeptides and require at least one terminus of the substrate to be in the active site of the enzyme. They can be further divided into endo- and exo-peptidases depending on whether they act in

the interior of the polypeptide or at a terminus. Proteases acting on intact proteins and not requiring carboxyl or amino termini are called proteinases. Sometimes endo-peptidases have also been called proteinases. In this dissertation, I will use "protease" as a general name for all enzymes involved in protein degradation.

Proteases (proteinases and endo-peptidases) are also classified according to their catalytic mechanisms, since their substrate specificities are often difficult to define (reviewed in Barrett, 1986; Neurath, 1984). Four classes have been defined by Hartley (1960); serine (EC 3.4.21), cysteine (EC 3.4.22), aspartic (3.4.23), and metalloproteases (3.4.24). These classes can be identified according to the effects of class-specific inhibitors on enzyme activity (Dunn, 1989; Salvesen and Nagase, 1989). This classification does not represent an evolutionary relationship. Thus, some classes contain so-called superfamilies, that is enzymes within a single class are derived from different origins.

Serine proteases comprise the most widespread and diverse group of proteases. This class contains two superfamilies; the trypsin and subtilisin families. Trypsin-like proteases have been found in both eukaryotes and prokaryotes. Subtilisin-like enzymes exist only in prokaryotes. The active site in serine proteases is a serine residue that attacks a carbonyl carbon of the substrate. These proteases are identified by their inhibition by phenylmethanesulfonyl fluoride (PMSF) or 3,4-dichloroisocoumarin.

Cysteine proteases are found in both eukaryotes and prokaryotes

and contain several superfamilies. The best characterized cysteine protease is papain from papaya (Carica papaya L.). Two superfamilies have been identified among bacteria. These are clostripain from Clostridium histolyticum (Weinberg and Sequin) Bergey et al. and proteases from Streptococcus spp. The active site of cysteine proteases involves a thiol group of cysteine, which can be inactivated by thiol-blocking agents such as iodoacetate.

Aspartic proteases have been detected only in eukaryotes and include pepsin involved in food digestion in higher animals. The catalytic site contains an aspartic residue, the carboxyl group of which acts on the substrate. Chemicals blocking the carboxyl group, such as pepstatin, inhibit aspartic proteases.

Metalloproteases are a very ancient group of enzymes from prokaryotes and eukaryotes (reviewed by Hofmann, 1985). They contain a metal ion, usually zinc, at the center of the active site, which is involved in the catalytic event. Calcium ions are often required for enzyme stabilization. Thermolysin from Bacillus thermoproteolyticus Rokko (Note that this bacterium is not described in any systematic list, but its origin is described by Endo [1962]) is a typical bacterial metalloprotease, while carboxypeptidases A and B, collagenases, and gelatinases represent mammalian metalloproteases. Although similar in size and catalytic activities, the bacterial and mammalian metalloproteases do not share sequence similarity, suggesting that the bacterial metalloproteases are evolutionarily distinct in origin. Inhibitors of metalloproteases include metal-chelating agents such as

EDTA and phenanthroline.

Production of extracellular proteases by phytopathogenic bacteria

Protease production by plant pathogens has received little attention. Yet, Riker et al. (1935) made the first reports of protease activity in diseased plant tissue and described gelatin and casein hydrolysis by Xanthomonas campestris pv. alfalfae (Riker et al.) Dye, the causal agent of bacterial leaf spot of alfalfa (Medicago sativa L.). It hydrolyzed gelatin and casein and was most active at pH 8.0 (Reddy et al., 1970). This enzyme was probably a metalloprotease, since calcium and zinc ions increased protease activity and EDTA or dialysis inhibited activity. Protease activity was greater in broths prepared from susceptible plants compared to broths prepared from resistant plants; however, the growth rates were equal in both broths. Protease activity on casein was reported in Pseudomonas syringae pv. lachrymans (Smith and Bryan) Young et al., both in culture and during pathogenesis in cucumber (Keen et al. 1967a; 1967b). Protease production by Pseudomonas fluorescens Migula has also been reported (Hagihara, 1960). There are few studies on the role of protease in plant pathogenesis. Sands and Hankin (1975) observed that protease production in plant pathogenic pseudomonads correlated more highly with soft rotting ability than did pectolytic enzyme production. The nutritional role of protease during in planta growth has been studied using protease-deficient mutants. Protease-deficient mutants of Xanthomonas campestris pv. malvacearum (Smith) Dye and Xanthomonas campestris pv. oryzae (Ishiyama) Dye have reduced in planta growth compared to the wildtype pathogen (Gholson, et

al., 1989; Xu and Gonzales, 1989).

Xanthomonas campestris pv. campestris (Xcc), causing black rot of crucifers, secretes several extracellular enzymes including proteases, pectate lyases, endoglucanases, and amylase. A protease-deficient mutant of Xcc obtained by Tn5 mutagenesis and marker exchange techniques was tested for pathogenesis on turnip (Brassica rapa L.) leaves and seedlings (Tang et al., 1987). The development of rotting symptoms caused by the protease-deficient mutant in variety 'Just Right' was not as rapid as that caused by the wildtype, whereas, on the variety 'Golden Ball' there was little difference. The growth rates and final population sizes in planta between the protease-deficient mutant and wildtype were similar. These results suggest a minor role for Xcc protease in black rot. The nucleotide sequence of this protease and its deduced amino acid sequence show a strong similarity with the subtilisin family of serine proteinases (Liu et al., 1990). PMSF, an inhibitor of serine proteases, inhibited its activity.

Protease activity of Ech strain 3937JS2, measured as azocasein hydrolysis, was detected in medium containing tryptone and yeast extract, but not in a minimal-glycerol medium (Wandersman et al., 1986). Due to protease instability, characterization of the proteases required development of an over-producing mutant, HP3. Two proteases were described with molecular masses of 50 and 55 kDa as estimated by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and skim milk-agar overlay. The major protein (50 kDa) had a pI of 5.8 and was inhibited by PMSF, but not by EDTA, suggesting that it was a serine

protease. The minor protease (55 kDa) had a pI of 4.6. Due to its instability, it was not further characterized. The same authors also studied proteases from a different Ech strain, B374, and its spontaneous protease over-producing mutant, HP1 (Wandersman et al., 1987). This mutant secretes three antigenically distinct proteases, A, B, and C with molecular masses of 50, 53, and 55 kDa, respectively. These proteases are also produced in E. coli transformed with a cosmid containing a 40 kb DNA fragment from B374. The gene encoding protease B was sequenced and showed significant sequence identity with the metalloprotease from Serratia marcescens Bizio (Delepelaire and Wandersman, 1989). Proteases A and C have not been sequenced. An extracellular protease is also produced by Ech strain EC16. Its protease is inhibited by EDTA, but not by PMSF or iodoacetate suggesting that it is a metalloprotease (Barras et al., 1986). The gene for this protease was cloned and transformed into E. coli strain HB101, but further description of it has not been reported.

Ecc proteases have been less studied than those from Ech. Friedman (1961) reported that Ecc strain 58-27 produced more protease than its avirulent, ultraviolet-induced protease-deficient mutant on gelatin and litmus milk. The growth of the wildtype compared to the mutant was up to 50% greater in minimal medium containing yeast extract. The author suggested that the protease may affect virulence by tissue degradation, accelerated protein synthesis, or by increased energy sources. Tseng and Mount (1973) partially purified a protease with a pI of 8.3 from Ecc strain EC14 using DEAE-cellulose and isoelectric

focusing. The protease did not cause tissue maceration or cellular death on cucumber and potato slices; however, cucumber protoplasts were lysed suggesting that the enzyme acts on membrane proteins. A protease with a pI of 4.8 also from strain EC14 has been cloned (Allen et al., 1986; Smith et al., 1987) and is the basis for my dissertation research.

Bacterial extracellular metalloproteases

Since the protease from Ecc strain EC14 was identified as a metalloprotease (Chapter II), I will concentrate here on this group of proteases. While several bacterial metalloproteases have been reported, the best characterized is the extracellular neutral protease, thermolysin, secreted by thermophilic B. thermoproteolyticus (Hofmann, 1985). This enzyme has a molecular mass of 34 kDa and contains one zinc and four calcium ions. The zinc and calcium ions have been located within the protein using chemical and X-ray diffraction analyses (Matthews et al., 1972a). The zinc atom involved in the catalysis is attached to three residues and is located near the center of the protein in a hydrophobic pocket believed to be the substrate binding site (Matthews et al., 1972a; 1972b). Calcium ions stabilizing the enzyme are situated near the substrate binding site and in flanking regions on either side. Although the amino acid sequence of thermolysin has been determined (Titani et al., 1972), the gene encoding thermolysin has not been cloned or sequenced.

Metalloproteases have been identified in several Bacillus spp. (Sidler et al., 1986; Takagi et al., 1985; Vasantha et al., 1984; Yang et al., 1984), in Pseudomonas cepacia Burkholder (McKevitt et al.,

1989), Pseudomonas aeruginosa (Schroeter) Migula (Morihara and Tsuzuki, 1975; Bever and Iglewski, 1988; Fukushima et al., 1989), S. marcescens (Nakahama et al., 1986), Ech (Barras et al., 1986; Delepelaire and Wandersman, 1989), V. cholerae (Booth et al., 1983), Legionella pneumophila Brenner et al. (Black et al., 1990; Dreyfus and Iglewski, 1986), and Streptomyces griseus (Krainsky) Waksman and Henrici (Trop and Birk, 1970). The gene encoding the P. aeruginosa metalloprotease, elastase, has been sequenced (Bever and Iglewski, 1988; Fukushima et al., 1989). Its deduced amino acid sequence was similar to thermolysin and L. pneumophila metalloprotease (Black et al., 1990). The metalloprotease from S. marcescens has less sequence identity to thermolysin (Nakahama et al., 1986). Catalytic residues are, however, conserved in all metalloproteases and can be identified in amino acid sequence comparisons (Bever and Iglewski, 1988). Differences observed in the substrate binding sites of the enzymes may suggest differences in substrate specificities. In general, metalloproteases bind specifically to hydrophobic amino acid residues at the amino-side of the cleavage site (Morihara and Tsuzuki, 1975). Thermolysin has a broad substrate specificity and may digest native proteins more completely than other proteases (Heinrikson, 1977). Metalloproteases are usually 30 to 40 kDa in size, except S. marcescens and Ech proteases (50 to 55 kDa), focus isoelectrically at pH 4.0 to 6.0, and have neutral pH activity optima.

Regulation of metalloproteases

Protease production is usually repressed early in the growth cycle and derepressed in the late exponential or early stationary phase.

Derepression may be due to increasing cAMP levels, since regulation by catabolite repression is commonly observed (Stinson and Merrick, 1974). Repression by tricarboxylic acid cycle intermediates such as succinate, malate, and α-ketoglutarate in a manner similar to catabolite repression has been reported (Boethling, 1975). Induction of protease by peptides and other proteinaceous substrates has been observed (Litchfield and Prescott, 1970), while degradation products, such as free amino acids, often repress protease synthesis, probably by feedback inhibition (May and Elliot, 1968).

Initiation of synthesis for some metalloproteases corresponds to limiting nutrients in the environment. Iron, for example, regulates elastase synthesis in some P. aeruginosa strains resulting in reduced elastase production in high iron concentrations (Bjorn et al., 1979).

Many metalloproteases destined for secretion are not active within the cell. In Ech, the lack of intracellular protease activity is due to a low molecular weight protein inhibitor (Wandersman et al., 1987). Alternatively, proteases are secreted as zymogens, and activated during transport. These mechanism may protect intracellular proteins from proteolysis by metalloproteases destined for secretion.

Metalloproteases in pathogenesis

As previously mentioned, information about the function of secreted metalloproteases in phytopathogens is very limited. In contrast, several extracellular metalloproteases have been identified and characterized in a wide range of animal and insect pathogenic bacteria including Bacillus spp. and Nocardia spp. (Zlotnik et al.,

1984), P. aeruginosa (Bever and Iglewski, 1988), V. cholerae (Booth et al.,1983; Honda et al., 1989), S. marcescens, and L. pneumophila (Dreyfus and Iglewski, 1986). Metalloproteases have been implicated in pathogenesis, since their production and virulence are correlated. This correlation is especially strong among opportunistic pathogens, such as P. aeruginosa and S. marcescens, with multiple pathogenicity factors (Morihara and Homma, 1985).

Proteases may facilitate colonization of host tissue, evasion of host defense response, or metabolism of host proteins for bacterial growth. Colonization of host tissue may be aided by protease, since metalloproteases cleave structural proteins. Elastase, a S. marcescens protease, and thermolysin cause necrosis of the cornea with destruction of structural elements (Molla et al., 1987). Elastin, proteoglucan, and collagen, major structural elements in all connective tissues, are degraded by elastase, L. pneumophila and S. marcescens proteases. Metalloproteases from S. marcescens and V. cholerae also cleave fibronectin, which maintains the structural network cross-linking epithelial/fibroblastic cells in connective tissue (Finkelstein et al., 1983; Molla et al., 1988). Proteases from erwinias are also able to degrade, albeit slowly, structural proteins such as HRGPs (Lewosc, et al., 1989). Since collagen and HRGPs resemble each other in their polyproline helical structure, it is possible that other metallo-proteases may also cleave HRGPs (Adair and Appel, 1989).

Metalloproteases affect mammalian defenses by inactivating immunoglobins (Molla et al., 1986). They also degrade proteinase inhibitors

(Molla et al., 1986), cytochrome-C peroxidases (Rönnberg, 1987), several components of complement factors, and certain lysozymes (Jacquot et al., 1985). Lysozymes are important for defense against microbes in egg yolk, tears, and lungs. The basic charge of lysozymes may aid substrate binding by acidic metalloproteases. A role in protection against complement-mediated killing or other serum bacteriocidal factors was also suggested for a metalloprotease produced by a fish pathogen, Aeromonas hydrophila (Chester) Stanier (Leung and Stevenson, 1988). A Tn5-induced, protease-deficient mutant of A. hydrophila was more susceptible to the bacteriocidal effects of trout serum than the wildtype bacteria.

Necrosis and cleavage of proteins by proteases in mammals cause secondary effects by activating several defense-related pathways such as the kallikrein-kinin system which affects vascular permeability. The cleavage of Hageman factor activates the kallikrein-kinin system leading to hemorrhage, edema, and inflammation. Several microbial proteases activate the kallikrein-kinin system including elastase, Vibrio vulnificus Reichelt et al. protease, and thermolysin (Miyoshi and Shinoda, 1988; Molla et al., 1987; 1989). Digestion of plant proteins by Ecc protease may cause similar cascade reactions in the host by activating or destroying induced host defense proteins such as lipid acyl hydrolase, chitinases, or peroxidases.

Besides their protein cleaving activities, many bacterial metalloproteases have cytotoxic effects. Proteases from S. marcescens are toxic to fibroblasts, which comprise connective tissues of lung and

corneal stroma (Molla et al., 1986). Cytotoxicity is correlated with protease activity (Maeda et al., 1987) and may be due to degradation products which may form toxic products, such as ammonia, phenols, indoles, and amines (Gibson, et al., 1989). The toxicity of Ecc protease on plant cells is unknown. Ecc culture supernatants have been reported to contain a toxic factor that kills plant tissues. Although several researchers have correlated cytotoxicity with pectic enzymes (Bateman and Basham 1976; Tseng and Mount, 1973), this may not be the case since cytotoxicity occurs even when the cells are plasmolyzed, a condition protecting plant cells from loss of cell wall integrity (Stephens and Wood, 1975). It is possible that the lysis of cucumber protoplasts observed by Tseng and Mount (1973) resulted from the cytotoxic effects of Ecc protease rather than cytoplasmic membrane protein degradation. Thermolysin and V. cholerae protease also cause hemagglutination of chicken erythrocytes (Booth et al., 1983), although this effect has not been demonstrated in vivo.

The nutritional effect of proteases has been discussed by Weinberg (1985). It is tempting to assume that amino acids and peptides resulting from host protein degradation are used as carbon and nitrogen sources for bacterial growth. Subsequently, the absence of protease may result in slower growth rate and reduced pathogenicity. This has been shown with a protease-deficient mutant of A. hydrophila (Leung and Stevenson, 1988). The mutant grew more slowly on host serum than the wildtype, whereas in minimal-glucose medium, in which protease production presents no advantage, their growth rates were the same.

There is some evidence that protease action also facilitates acquisition of limiting nutrients such as iron. The siderophore from P. aeruginosa, a pyoverdin, is able to release iron from human transferrin only in the presence of elastase (Döring et al., 1988). Protease from S. marcescens also degrades transferrin very slowly (Molla et al., 1986). Protease from V. cholerae cleaves another host iron-binding protein, lactoferrin (Finkelstein et al., 1983). Ech (Expert and Toussaint, 1985) and Ecc (Loper and Ishimaru, 1989) also produce siderophores, but the effect of proteases in releasing the iron from plant siderophores or other iron-containing compounds such as cytochromes is unknown.

It is interesting that metalloproteases alone are not able to cause all disease symptoms; thus, other virulence factors produced by the bacteria must be required. This suggests that metalloproteases are only one among several factors increasing pathogen virulence. In potato soft rot, pectolytic enzymes may release proteinaceous material via degradation of pectic substances in the primary cell wall. This may induce protease synthesis and facilitate its access to the cytoplasmic membrane and eventually to cytosolic and vacuolar material. Whether protease action is directed specifically against host structural and/or defense proteins, or occurs randomly, acting primarily as a nutritional factor, remains to be elucidated.

OBJECTIVES AND SIGNIFICANCE OF THE DISSERTATION RESEARCH

Development of pathogenesis results from a complex interaction between the host and the pathogen. So far, the mechanism of this

interaction between the soft rot pathogen, Ecc, and potato is not well understood. This is partly because not all the genetic determinants in pathogenesis are known. My study is intended to elucidate the role of extracellular protease in soft rot pathogenesis. The specific objectives are:

(1) To characterize the Ecc protease by subcloning and sequencing the prt gene encoding the protease; purifying the protein; analyzing it for molecular weight, pI, and sensitivity to chemical protease inhibitors; and comparing this information to the characteristics of known proteases.

(2) To determine the role of Ecc protease in potato soft rot by creating a protease-deficient Ecc mutant, assaying the mutant for its macerating ability and growth in planta, and determining when prt mRNA appears during pathogenesis. The prt expression data will be compared with data for expression of other Ecc genes encoding cell degrading enzymes and potato genes encoding protease inhibitors and HRGPs. This comparison will indicate if there is any correlation between the appearance of host defense factors and the appearance of Ecc Prt.

The expanding information about metalloproteases will be helpful in understanding the function of extracellular proteases in pathogenesis. Specifically, elucidating the function of Ecc protease will increase our knowledge of Ecc in soft rot interaction. For the future, it is hoped that better understanding of the molecular mechanism of potato soft rot on both the bacterial and plant sides will allow development of control measures against soft rot such as biological

control using disarmed Ecc strains or engineering more resistant potato varieties.

II. ERWINIA CAROTOVORA SUBSP. CAROTOVORA EXTRACELLULAR PROTEASES: CHARACTERIZATION AND NUCLEOTIDE SEQUENCE

To be submitted to **Journal of Bacteriology** by S.M.R. Kyöstiö, C.L. Cramer, and G.H. Lacy

ABSTRACT

The prt1 gene encoding extracellular protease from Erwinia carotovora subsp. carotovora (Ecc) strain EC14 in cosmid pCA7 was subcloned to create plasmid pSK1. The nucleotide sequence of the insert in pSK1 (1,878 bp) revealed a 1,041 bp open reading frame (ORF1) that correlated with protease activity in deletion mutants. ORF1 encodes a polypeptide of 347 amino acids with a calculated molecular mass of 38,826 Da. Escherichia coli transformed with pSK1 or pSK23, a subclone of pSK1, produces a protease (Prt1) intracellularly with a molecular mass of 38 kDa and pI of 4.8. Prt1 activity was inhibited by phenanthroline suggesting that it is a metalloprotease. The prt1 promoter was localized between 173 and 1,173 bp upstream of ORF1 by constructing transcriptional lacZ fusions. Primer extension identified the prt1 transcription start site 205 bp upstream of ORF1. The deduced amino acid sequence of ORF1 showed significant sequence identity to metalloproteases from Bacillus thermoproteolyticus (thermolysin), Bacillus subtilis (neutral protease), Legionella pneumophila (metalloprotease), and Pseudomonas aeruginosa (elastase). It has less sequence similarity to metalloproteases from Serratia marcescens and

Erwinia chrysanthemi. Locations for three zinc ligands and the active site for Ecc protease were predicted from thermolysin.

INTRODUCTION

Erwinia carotovora subsp. carotovora (Jones) Bergey et al. (Ecc) strain EC14 is a Gram-negative bacterium which causes soft rot on many plant species (Pérombelon and Kelman, 1980). Soft rot of potato (Solanum tuberosum L.) tubers is associated with the production of several degradative enzymes secreted by Ecc, including pectolytic enzymes, cellulases, proteases, and phospholipases (Collmer and Keen, 1986; Tseng and Mount, 1973). Pectolytic enzymes probably play the most important role in maceration by degrading pectic components of the plant cell wall and middle lamella, resulting in separation of the cells (Bateman and Basham, 1976). Possible roles for other degradative enzymes have not been established.

Several erwinias and pseudomonads causing soft rot secrete proteases. Among soft rot pseudomonads, extracellular protease production correlates more strongly with the ability to macerate plant tissue than does pectolytic enzyme production (Sands and Hankin, 1975). In Ecc, large amounts of extracellular protease are produced when grown in rich broth, on bean (Phaseolus vulgaris L.) hypocotyls, or sliced cucumber (Cucumis sativus L.) fruit (Tseng and Mount, 1973), but its physiological role is unknown. Protease may aid in the degradation of plant cell wall components, cytoplasmic membranes, or cytosolic proteins. Purified Ecc protease causes limited cell death on cucumber

disks and lysis of cucumber protoplasts (Tseng and Mount, 1973). The release of amino acids and small peptides by Ecc protease may increase the rate of bacterial growth, thereby increasing the ability of the pathogen to colonize its host. The nutritional benefit derived from the proteolytic digestion of host macromolecules may contribute to greater virulence (in the sense of causing greater host damage) of protease-producing human pathogens including Pseudomonas aeruginosa (Schroeter) Migula, Staphylococcus aureus Rosenbach, and Vibrio cholerae Pacini (Weinberg, 1985).

A cosmid (pCA7) encoding extracellular protease (Prt1) was previously detected in an Ecc genomic library and complemented a Tn5-induced, protease-deficient mutant, L-763, of Ecc (Allen et al., 1986). Here we report subcloning and sequencing of the protease gene (prt1) from pCA7. To clarify the function of the protease encoded by prt1 we have further constructed an Ecc marker exchange mutant for prt1, since Southern analysis of protease-deficient mutant L-763 indicated that prt1 was not interrupted by Tn5 (Appendix B). Our results show that the DNA sequence of prt1 is most similar to Bacillus thermoproteolyticus Rokko thermolysin (Titani et al., 1972); prt1 shows little sequence relatedness to metalloproteases of Erwinia chrysanthemi Burkholder et al., a closely related soft rot pathogen (Dahler et al., 1990; Delepelaire and Wandersman, 1989).

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and chemicals

Erwinia carotovora subsp. carotovora strain EC14 and cosmid pCA7 have been described previously (Allen et al., 1986; Roberts et al., 1986). Escherichia coli (Migula) Castellani and Chalmers strains DH5α (Bethesda Research Laboratories [BRL], Inc., Gaithersburg, Md.) and CB806 (Schneider and Beck, 1987) were used as plasmid hosts. Plasmids pSK- (BlueScript; Stratagene, La Jolla, Ca.), pBR322 (BRL), and pUC4-KIXX (Pharmacia, Inc., Piscataway, N.J.) were used for cloning. Plasmid pCB267 contains a promoterless lacZ gene (Schneider and Beck, 1987) useful for detecting promoter activity. Bacteria were grown in LB broth (Difco Inc., Detroit, Mi.), on LB agar (Difco) containing 100 μg/ml ampicillin or 10 μg/ml tetracycline, in protein extraction medium (PEM: 0.1% polygalacturonic acid, 0.5% tryptone, and 0.5% yeast extract), or on Davis' minimal agar (Lederberg, 1950). Gelatin plates (Hankin and Anagnostakis, 1975) were used to detect Ecc protease production in E. coli transformed with plasmids. Chemicals, antibiotics, and dyes were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless stated otherwise.

Isoelectric focusing

Isoelectric focusing (IEF) gels were prepared and run by the method of Ried and Collmer (1985) except that the ampholyte concentration was modified; 0.4 ml of ampholytes pH 3 to 10 (Servalyte; Serva Fine Chemicals, Haake Buchler Instruments, Inc., Saddle Brook, N.J.) and 0.9 ml of ampholytes pH 4 to 6 (Servalyte) were used. Intra-

and extracellular protein samples for IEF were prepared from 5 ml overnight cultures grown in LB at 37°C with orbital shaking (100 rpm). Turbidity at 600 nm was measured for each culture. Cultures were centrifuged for 2 min at 1,000 x g to pellet the cells; pellets and supernatants were saved. Each culture supernatant (2.0 ml) was concentrated five-fold using microconcentrators (exclusion limit 10 kDa; Centricon-10; Amicon Corp., Danvers, Ma.). For extracellular proteins, concentrated supernatants were standardized to the same original culture turbidity and applied (10 to 15 µl) on the IEF gel. For intracellular proteins, cell pellets were washed twice with 0.05 M Tris-HCl (pH 8.0), resuspended in 250 µl of the same buffer, and one drop of toluene was added to lyse the cells. The mixture, in capped tubes, was vortexed for 30 sec and then allowed to stand open to the atmosphere for 30 min to evaporate the toluene. Protein concentrations for cell lysates were determined by the Bradford (1976) method (BioRad, Richmond, Ca.). Equal amounts of protein (4.2 µg) were applied on an IEF gel.

Protease activity in IEF gels was localized using a 0.35 mm gelatin overlay (4% gelatin and 1% agarose in 50 mM Tris-HCl [pH 8.0]). The overlay was cast on plastic support (Agarose Gelbond; FMC BioProducts, Rockland, Ma.; Ried and Collmer, 1985) and incubated in contact with the acrylamide gel at 37°C from 1 h to overnight. Protease activity was detected by submerging the overlay in $HgCl_2$ (12 g in 80 ml water and 16 ml conc. HCl), which causes a cloudy white gelatin precipitation. Clear zones indicate areas where protease has degraded the gelatin.

Protease inhibitor assays

3,4-Dichloroisocoumarin (3,4-DCI), ethylenediaminetetraacetic acid (EDTA), iodoacetate, pepstatin, phenanthroline, phenylmethanesulfonyl fluoride (PMSF), and phosphoramidon were tested for their ability to inhibit protease activity (Salvesen and Nagase, 1989). Protease was preincubated with each inhibitor for 20 min at 37°C before assaying for activity. Protease activity was measured by azocasein degradation according to Reckelhoff et al. (1985) with the following modifications: the reaction was started by adding 125 µl of prewarmed 2% azocasein (37°C) to 125 µl of enzyme preparation in 10 mM Tris-HCl (pH 8.0) containing 2 mM $CaCl_2$ followed by static incubation for 20 min at 37°C. The reaction was stopped by adding 1 ml of 5% trichloroacetic acid. The mixture was centrifuged for 2 min at 1,000 x g at room temperature and the $A_{340\ nm}$ of the supernatant measured. One unit (U) of protease activity was defined as an absorbance increase of 0.001 min^{-1}.

ß-galactosidase assays

Expression of ß-galactosidase was detected on LB plates plus ampicillin spread with 100 µl of a 20 mg/ml solution of indolyl-ß-galactoside (BRL). Enzyme assays for ß-galactosidase activity were performed according to Miller (1972).

DNA procedures

Procedures for agarose gel electrophoresis, restriction enzyme analysis, and ligation were performed using standard methods (Maniatis et al., 1982). Plasmid DNA used for cloning and sequencing was isolated

from 5 ml LB cultures using an alkaline lysis method (Ish-Horowicz and Burke, 1981). DNA fragments for cloning were separated by electrophoresis in 0.7% (Sea Plaque; FMC BioProducts) or 1.0% (NuSieve; FMC BioProducts) low-melting point agarose gels and the DNA from the excised gel bands was used for ligation (Struhl, 1986). Recombinant plasmids were transformed into E. coli by the method of Hanahan (1983). Restriction enzymes, T4 ligase, exonucleases III and VII, and Klenow fragment of DNA polymerase I were purchased from BRL. Alkaline phosphatase was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.).

DNA sequencing and analysis

Unidirectional deletions of pSK1 and pSK2 were prepared by cleaving the plasmids with ApaI and ClaI within the multiple cloning site of pSK- to create 3' and 5' overhangs, respectively, followed by digestion of the 3' recessed strand with exonuclease III (Henikoff, 1984). Single-stranded DNA was removed by digestion with exonuclease VII, the blunt ends created by the Klenow fragment, ligated, and transformed into E. coli. The approximate location of prt1 within pSK1 and pSK2 was determined by screening deletion mutants for Prt1 activity on gelatin plates. Selected mutants were sequenced by the Sanger dideoxy-chain termination method (Sanger et al., 1977) using a kit (Sequenase; United States Biochemicals Corp., Cleveland, Oh.) and [^{35}S]dATP (NEN Research Products, DuPont Company, Wilmington, Del.). Sequence data were analyzed using the University of Wisconsin Genetics Computer Group 1989 software (Devereux et al., 1984).

Primer extension

Ecc was grown in PEM at 30°C with orbital shaking (100 rpm) to early and late stationary phases (turbidity at 600 nm = 0.9 and 1.4, respectively). Total cellular RNA for primer extension analysis was isolated according to the method of Szumanski and Boyle (1990). The transcription start site of prt1 was determined using a 22-nucleotide-long synthetic oligonucleotide (5'-GGTGCCGTTTGCGATAATACGA-3') complementary to prt1 synthesized by Dr. M. Lederman (Department of Biology, Virginia Polytechnic Institute and State University [VPI&SU], Blacksburg, Va.). The primer extension protocol was obtained from Dr. R. Moore (Department of Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, VPI&SU). Approximately 20 to 40 µg RNA and 40 ng primer were ethanol-precipitated together overnight at 20°C. After centrifugation for 45 min at 10,000 x g at 4°C, the pellet was dissolved overnight in 12.5 µl diethylpyrocarbonate-treated water. The primer was annealed by heating the mixture at 100°C for 90 sec and slowly cooling it to room temperature. The primer extension reaction was performed in reverse transcriptase assay buffer (50mM Tris-HCl [pH 8.0], 6 mM $MgCl_2$, 40 mM KCl, and 10 mM DTT). One mM each of dCTP, dGTP, and dTTP; 1 µl [^{35}S]-dATP (1,320 Ci/mmol, 10.4 mmol/ml); and 40 U of reverse transcriptase (Boehringer-Mannheim) were added to the mixture in a total volume of 22 µl, and incubated at 42°C for 20 min. The reaction was chased by adding 1 mM of all deoxynucleotides and incubating an additional 30 min. Three µl of the mixture was mixed with 1 µl of stop-dye from the Sequenase kit and run on a 7% (w/v) acrylamide sequencing

gel. A sequencing reaction performed using the same primer and pSK1 was used to determine the transcription start site.

Northern analysis

RNA was isolated from Ecc grown in glycerol broth or *in planta* and the *prt1* mRNA expression was measured by Northern analysis as previously described (Yang et al., 1989). A ^{32}P-labelled 1 kb *Sma*I-*Eco*RI fragment of pSK1 was used as a *prt1* probe. An RNA ladder (BRL) was used to determine the molecular size of the RNA transcript.

Bacterial mating, plasmid curing, and marker exchange

Plasmids with *prt1* inactivated by inserting a gene for kanamycin resistance were mated into Ecc and marker exchange for wildtype *prt1* was forced by phosphate starvation. Triparental mating was performed using a helper plasmid pRK2013 (Ditta et al., 1980). Bacterial strains (Ecc, *E. coli* HB101/pRK2013, and *E. coli* DH5α/pSK16 containing inactivated *prt1*) grown separately in 50 ml LB medium with orbital shaking (100 rpm) at 30°C overnight were impacted by filtration on membranes (0.45 μm pore size) and incubated on LB plates at 30°C overnight. Bacteria were washed from membranes with sterile deionized water, diluted in serial manner, plated on Davis' minimal agar containing 30 μg/ml kanamycin, and incubated at 30°C for 48 hrs. Plasmid transfer was confirmed for single Kan^r colonies by plasmid isolation, electrophoresis in agarose, and visualization by ethidium bromide fluorescence in ultraviolet light. To cure plasmids and force marker exchange (Roeder and Collmer, 1985), bacteria were grown in A-P phosphate starvation medium (Torriani, 1960) containing 30 μg/ml kanamycin. Loss of plasmids and Prt1$^-$ phenotype

were confirmed by agarose gel electrophoresis and assay on gelatin plates, respectively.

Southern hybridization

Chromosomal DNA was isolated according to Ausubel et al. (1989), digested with EcoRI, and fragments were separated electrophoretically on 0.7% agarose. DNA was transferred to a nylon membrane (Nytran; Schleicher and Schuell, Inc., Keene, NH) according to Maniatis et al. (1982). Probe labelling, hybridizations, washings, and detection were carried out following the manufacturer's instructions (Genius System, Boehringer-Mannheim, Indianapolis, Ind.).

RESULTS

Subcloning of prt1

BglII digested pCA7 (Allen et al., 1986) into six fragments; an 8.0 kb BglII fragment cloned into the BamHI site of pSK- (pSK10) expressed protease activity (Prt1) in E. coli DH5α. Plasmid pSK10 was digested with HpaI and HindIII to delete a 3.0 kb fragment. The remaining DNA, containing a 5.0 kb BglII-HpaI insert from Ecc and the pSK- vector, was purified from an agarose electrophoresis gel, blunt ends were made with Klenow fragment, and religated to create pSK2 (Fig. 1). To clone the 5.0 kb BglII-HpaI fragment in the opposite orientation in pSK-, pCA7 was digested with BglII and HpaI, the fragments were treated with Klenow fragment, and the gel-purified BglII-HpaI fragment was ligated into SmaI-cleaved pSK- to produce pSK1 (Fig. 1). Prt1 activity on gelatin plates in E. coli transformed with pSK1 or pSK2

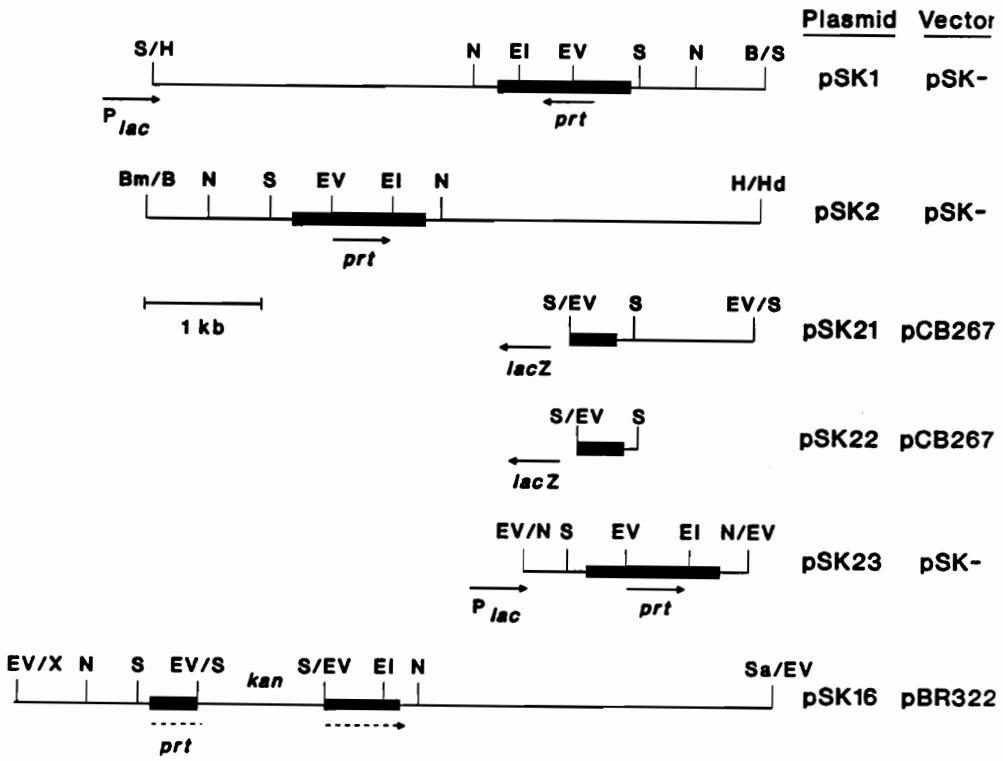


Fig. 1. Endonuclease restriction site maps for inserts of E. carotov subsp. carotovora (Ecc) DNA in plasmids pSK1, pSK2, pSK21, pSK22, pSK and pSK16. The Ecc protease gene (prt1) (thick line), directions of transcription (arrows: solid, functional gene; dashed, nonfunctional), lacZ promoters in pSK- vector (P), ß-galactosidase genes (lacZ), inserted kanamycin resistance gene (kan), and digestion sites for endonucleases BamHI (Bm), BglII (B), EcoRI (EI), EcoRV (EV), HindIII (Hd), HpaI (H), NruI (N), SalI (Sa), SmaI (S), and XbaI (X) are indicated. Double restriction endonuclease sites are results of blun end ligations and are not functional. The construction of the plasmi is described in Results.

indicated that the 5.0 kb BglII-HpaI fragment contained the prt1promo as well as the open reading frame.

Ecc and E. coli strain DH5α containing pCA7, pSK1, or pSK2 expressed a protease with a pI of 4.8 (Fig. 2). Occasionally, a seco band, probably due to protease degradation, was detected at pI 5.8. Most of the Prt1 activity for E. coli DH5α/pSK1 or /pSK2 was detected the intracellular fraction. No protease activity was detected for E. coli DH5α transformed with vector pSK-. Escherichia coli DH5α/pSK2 h approximately two-fold more protease activity than E. coli DH5α/pSK1 estimated from the size of the clearing zone in the gelatin overlay. This increase was probably due to synergistic transcriptional fusion between prt1 and the lacZ promoter in the pSK- vector.

Nucleotide sequence of prt1

The location of prt1 in the 5.0 kb BglII-HpaI fragment of pSK1 determined by testing deletion mutants for Prt1 activity (Fig. 3). A 1,878 bp region of pSK1 was sequenced for both DNA strands using overlapping deletion plasmids. A single 1,041 bp open reading frame (ORF1) was found between bases 663 and 1,703 (Fig. 4) that correlated with Prt1 activity of the deletion clones. The deduced polypeptide o ORF1 contained 347 amino acids with a calculated molecular mass of 38,826 Da. A putative Shine-Delgarno (ribosome binding) sequence, AGGAGA, was 7 to 12 bp upstream of the ATG (Met) initiation codon. A bp palindromic sequence was located 14 to 39 bases downstream from a termination codon (TGA). This sequence has the potential to form a stem-loop structure with a nine-nucleotide-long stem, with one mismat

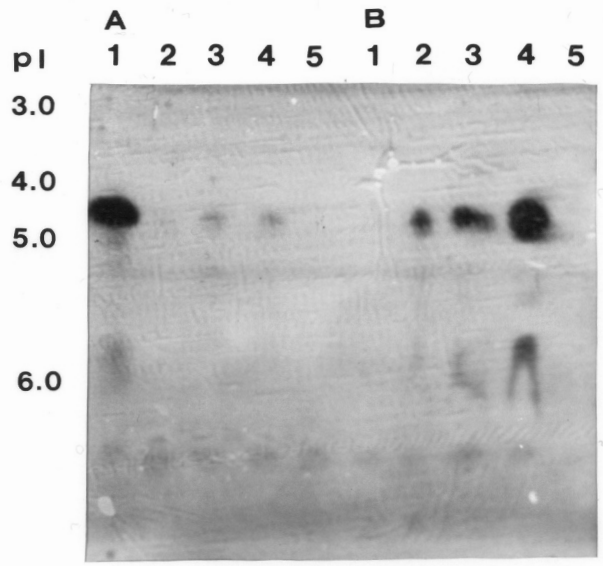


Fig. 2. Protease activity for E. carotovora subsp. carotovora (lane 1 and E. coli DH5α transformed with pCA7 (lane 2), pSK1 (lane 3), pSK2 (lane 4), or pSK- (lane 5) determined by isoelectric focusing followed by gelatin overlay. A. Extracellular fractions. B. Intracellular fractions. Loading sites at the bottom of the figure are visible in a lines.

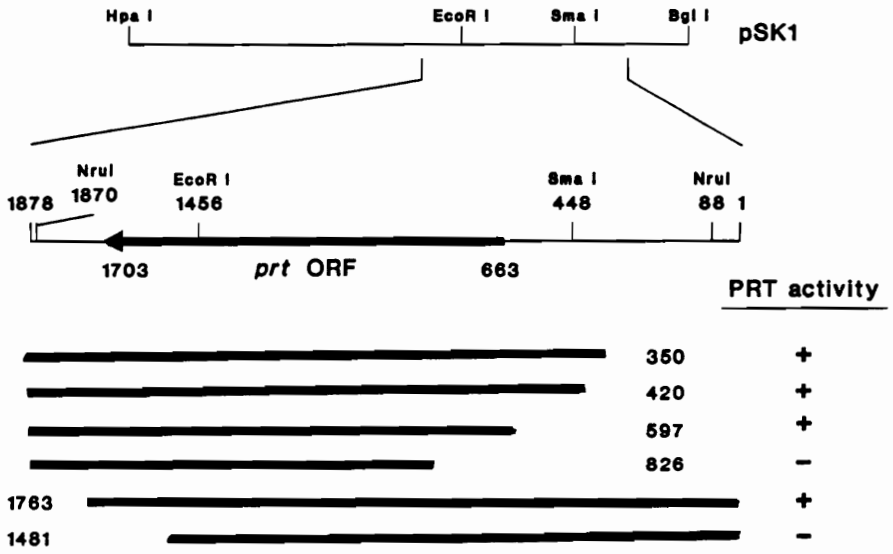
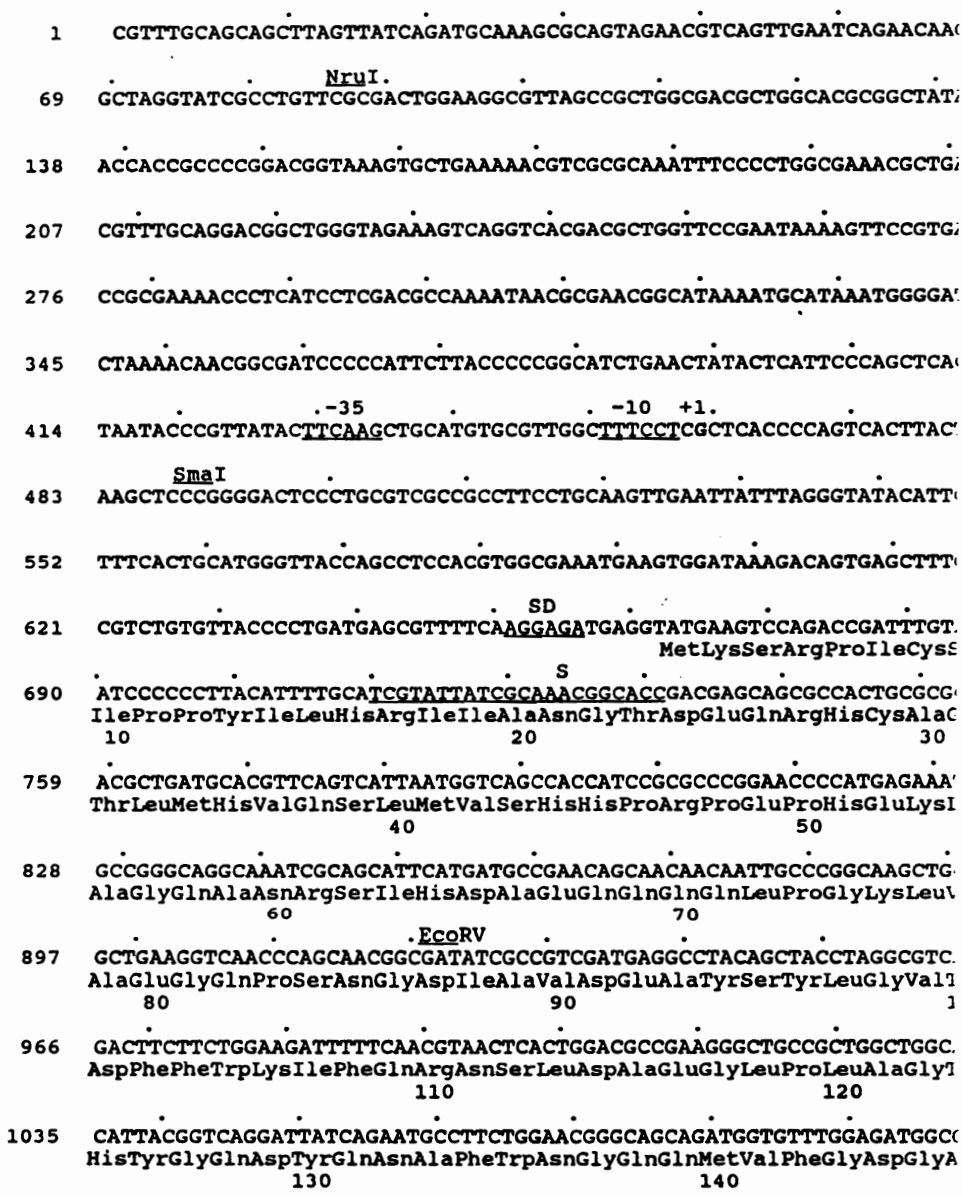


Fig. 3. Localization of the E. carotovora subsp. carotovora protease gene (prt1) by protease activity of deletion mutants. The open readi frame determined by sequencing (Fig. 4) is indicated by an arrow with starting and ending basepairs (bp) indicated within the sequenced reg (1,878 bp). Regions contained in deletion plasmids are shown with th bars. Numbers indicate the extent of the deletions to the nucleotide sequence in bp. Protease (PRT) activity (+/-) of the mutants was determined on gelatin plates. BglII, EcoRI, HpaI, NruI, and SmaI endonuclease digestion sites are indicated.


1 CGTTTGCAGCAGCTTAGTTATCAGATGCAAAGCGCAGTAGAACGTCAGTTGAATCAGAACAA
NruI
69 GCTAGGTATCGCCTGTTCGCGACTGGAAGGCGTTAGCCGCTGGCGACGCTGGCACGCGGCTAT
138 ACCACCGCCCCGGACGGTAAAGTGCTGAAAAACGTCGCGCAAATTTCCCCTGGCGAAACGCTG
207 CGTTTGCAGGACGGCTGGGTAGAAAGTCAGGTCACGACGCTGGTTCCGAATAAAAGTTCCGTG
276 CCGCGAAAACCCTCATCCTCGACGCCAAAATAACGCGAACGGCATAAAATGCATAAATGGGGA
345 CTAAAACAACGGCGATCCCCCATTCTTACCCCCGGCATCTGAACTATACTCATTCCCAGCTCA
-35 -10 +1
414 TAATACCCGTTATACTTCAAGCTGCATGTGCGTTGGCTTTCCTCGCTCACCCCAGTCACTTAC
SmaI
483 AAGCTCCCGGGGACTCCCTGCGTCGCCGCCTTCCTGCAAGTTGAATTATTTAGGGTATACATT
552 TTTCACTGCATGGGTTACCAGCCTCCACGTGGCGAAATGAAGTGGATAAAGACAGTGAGCTTT
SD
621 CGTCTGTGTTACCCCTGATGAGCGTTTTCAAGGAGATGAGGTATGAAGTCCAGACCGATTTGT
MetLysSerArgProIleCys
S
690 ATCCCCCCTTACATTTTGCATCGTATTATCGCAAACGGCACCGACGAGCAGCGCCACTGCGCG
IleProProTyrIleLeuHisArgIleIleAlaAsnGlyThrAspGluGlnArgHisCysAlaC
10 20 30
759 ACGCTGATGCACGTTCAGTCATTAATGGTCAGCCACCATCCGCGCCCGGAACCCCATGAGAAA
ThrLeuMetHisValGlnSerLeuMetValSerHisHisProArgProGluProHisGluLysI
40 50
828 GCCGGGCAGGCAAATCGCAGCATTCATGATGCCGAACAGCAACAACAATTGCCCGGCAAGCTG
AlaGlyGlnAlaAsnArgSerIleHisAspAlaGluGlnGlnGlnGlnLeuProGlyLysLeuV
60 70
EcoRV
897 GCTGAAGGTCAACCCAGCAACGGCGATATCGCCGTCGATGAGGCCTACAGCTACCTAGGCGTC
AlaGluGlyGlnProSerAsnGlyAspIleAlaValAspGluAlaTyrSerTyrLeuGlyVal
80 90
966 GACTTCTTCTGGAAGATTTTTCAACGTAACTCACTGGACGCCGAAGGGCTGCCGCTGGCTGGC
AspPhePheTrpLysIlePheGlnArgAsnSerLeuAspAlaGluGlyLeuProLeuAlaGly
110 120
1035 CATTACGGTCAGGATTATCAGAATGCCTTCTGGAACGGGCAGCAGATGGTGTTTGGAGATGGC
HisTyrGlyGlnAspTyrGlnAsnAlaPheTrpAsnGlyGlnGlnMetValPheGlyAspGlyA
130 140

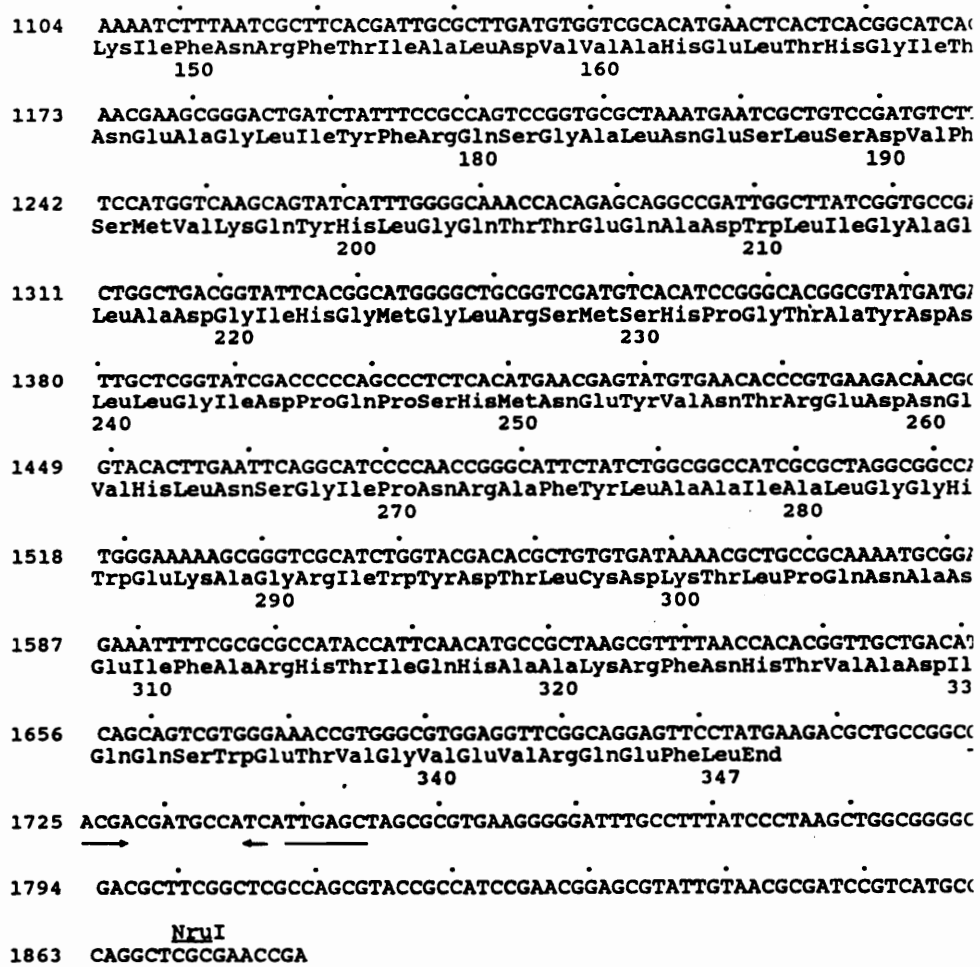


Fig. 4. Nucleotide and deduced amino acid sequence of the E. carotov subsp. carotovora protease gene (prt1). Indicated are those sequence most closely corresponding to the E. coli consensus sequences for the Pribnow box (-10), the recognition site (-35), ribosome binding site (SD), transcription initiation site (+1), the putative cleavage sites for signal peptidase (S), restriction endonuclease sites (NruI, SmaI, and EcoRI, and EcoRV), and the putative transcription terminator (horizontal arrows; break in arrow represents a mismatch in the inver repeat). Underlined within the open reading frame is the location of the sequence complementary to the primer used for primer extension (F 5). The nucleotide sequence for prt1 was submitted to GeneBank and assigned accession number M36651.

and an eight-nucleotide loop. Since the stem is not followed by a T-rich sequence, it is not clear if this structure is involved in transcription termination.

Localization of prt1 promoter

Promoter activity of regions upstream of ORF1 was tested by constructing transcriptional ϕ(prt1'-lacZ) fusions to the promoterles lacZ gene in plasmid pCB267. Plasmid pSK1 was digested with EcoRV or SmaI and EcoRV. A gel-purified 1.43 kb EcoRV restriction fragment containing 1,173 bp upstream of ORF1 was cloned into the SmaI site of pCB267 to create pSK21 (Fig. 1). Plasmid pSK22 was created by clonin 0.43 kb SmaI-EcoRV restriction fragment containing 173 bp upstream of ORF1 into the SmaI site of pCB267 (Fig. 1). Escherichia coli CB806/pSK21 harvested during late logarithmic phase (turbidity at 600 = 0.8) produced 41 U of ß-galactosidase; E. coli CB806/pSK22 expresse no ß-galactosidase activity. This confirms that the prt1 promoter and/or sequences affecting prt1 transcription lie between 173 and 1,1 bp upstream of OFR1. Escherichia coli CB806/pSK21 harvested at stationary phase (turbidity at 600 nm = 1.3) produced about ten-fold more ß-galactosidase activity (500 U) than did cells harvested in lat logarithmic phase. No activity was observed in E. coli DH5α/pSK22 ce harvested in the stationary phase.

Primer extension located the start of the prt1 mRNA 205 bp upstream of ORF1 (Fig. 5). The transcript was detected when RNA was isolated from Ecc during late stationary growth phase (turbidity at 6 nm = 1.4), but not at the early stationary phase (turbidity at 600 nm

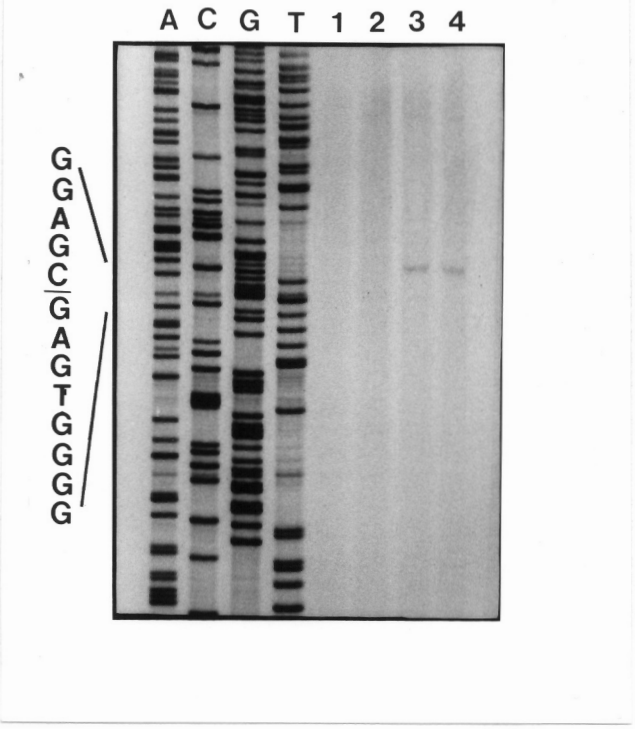


Fig. 5. Determination of the transcriptional start site for the E. carotovora subsp. carotovora (Ecc) protease gene (prt1) by primer extension. The sequencing reactions (A, C, G, and T) and primer extensions were initiated from primer (see Fig. 4) complementary to prt1. Primer extension reactions (lanes 1 to 4) contained: lane 1, 20 µg RNA from the early stationary growth phase; lane 2, 40 µg RNA from the early stationary phase; lane 3, 20 µg RNA from the late stationary phase; and lane 4, 40 µg RNA from the late stationary phase. The base sequence on the left (complementary to that shown in Fig. 4) indicates the first deoxynucleotide, cytosine (underlined) inserted in the prt1 transcript.

0.9). The putative promoter sequences, TTTCCT and TTCAAG at -7 and -29, respectively, are separated by 16 bp (Fig. 4). These sequences do not resemble strongly the E. coli -10 (TATAAT) and -35 (TTGACA) consensus promoter sequences (Hawley and McClure, 1983).

The size of the prt1 transcript, 1.3 kb, was estimated from a Northern blot using total RNA isolated from in planta grown Ecc (Fig. 6). This size is consistent with the length of the ORF1 (1,041 bp) plus 205 bp upstream and 40 to 50 bp downstream.

Sequence comparisons with other proteases

The DNA and predicted amino acid sequences for Prt1 were used to search the GenEMBL and NBRF databases in the University of Wisconsin Genetics Computer Group sequence analysis software (Devereux et al., 1984). Prt1 was found to be similar to several bacterial Zn-metalloproteases, including B. thermoproteolyticus thermolysin (Titani et al., 1972), P. aeruginosa elastase (Bever and Iglewski, 1988; Fukushima et al., 1989), Bacillus subtilis (Ehrenberg) Cohn neutral protease (Vasantha et al., 1984), Bacillus cereus Frankland and Frankland metalloprotease (Sidler et al., 1986), Bacillus amyloliquefaciens Fukumoto neutral protease (Yang et al., 1984), Bacillus stearothermophilus Donk metalloprotease (Takagi et al., 1985), Legionella pneumophila Brenner et al. metalloprotease (Black et al., 1990), E. chrysanthemi protease B (Delepelaire and Wandersman, 1989), and Serratia marcescens Bizio neutral protease (Nakahama et al., 1986). Amino acid sequences conserved among these metalloproteases were found in the central part of each enzyme. Two regions of Prt1, from residues

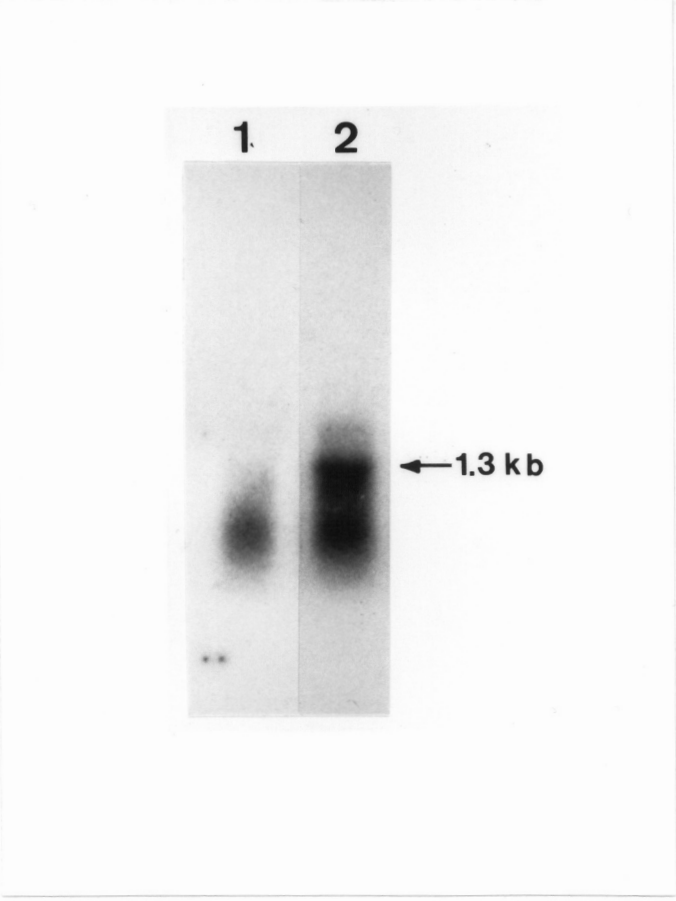


Fig. 6. Expression of prt1 mRNA of E. carotovora subsp. carotovora. Total bacterial RNA was isolated from glycerol broth-grown cells (lane 1) or 15 h after inoculation on sliced potato tubers (lane 2) at 30°C. Ten µg RNA was separated electrophoretically on a 1.2% agarose gel, transferred onto a Nytran membrane, and hybridized to ^{32}P-labelled prt1 probe (1.0 kb EcoRI-SmaI fragment [Fig. 1]). The molecular size of the prt1 transcript (1.3 kb) was estimated by comparison with RNA markers (RNA ladder; BRL) and is indicated on the right.

157 to 193 and from residues 258 to 276 were 58 to 71% and 42 to 74% identical, respectively, to proteases from B. thermoproteolyticus, B. subtilis, L. pneumophila, and P. aeruginosa (Fig. 7). Proteases from E. chrysanthemi and S. marcescens had only 18 to 32% and 21 to 26% similarity, respectively.

Characterization of Prt1

The DNA sequence information was used to find a restriction fragment containing only the prt1 ORF1; a 1.78 kb NruI fragment from pSK1 was cloned into the EcoRV site of pSK- to produce pSK23 (Fig. 1). Escherichia coli DH5α/pSK23 produced a 38 kDa protein with a pI of 4.8, which resembles the values calculated for the deduced protein from ORF1. Prt1 activity in the intracellular fraction of E. coli/pSK23 was inhibited by phenanthroline and phosphoramidon, but not by PMSF, iodoacetate, or pepstatin confirming that it is a metalloprotease (Table 1).

Marker exchange mutagenesis of prt1

The prt1 in plasmid pSK2 was insertionally inactivated by cloning a 1.2 kb SmaI fragment containing the kan gene from pUC4-KIXX into the EcoRV site within ORF1 (Fig. 1). The resulting plasmid, pSK15, was transformed into E. coli DH5α and its Prt1$^-$ phenotype was confirmed on gelatin plates. To ensure that no vector-derived regions of homology remained, the pSK15 insert was cloned into pBR322; pSK15 was digested with XbaI and SalI, the gel-purified 6.2 kb XbaI-SalI fragment was filled-in using Klenow fragment and ligated into the EcoRV site of pBR322. The resulting E. coli DH5α transformants with this 10.6 kb

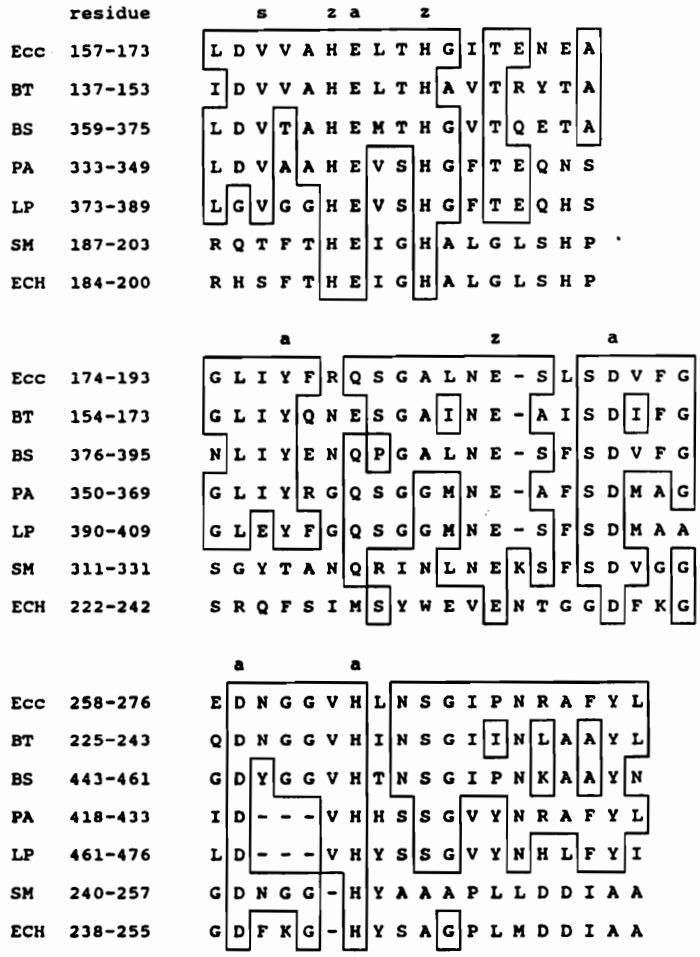


Fig. 7. Comparison of portions of the deduced amino acid sequence of the E. carotovora subsp. carotovora (Ecc) protease gene (prt1) to similar regions from other bacterial metalloproteases. Numbers refer to the location of the residues in the deduced amino acid sequence for each gene. Gaps (-) have been introduced to achieve optimal alignments. Residues involved in zinc binding (z), substrate binding (s), and the active site (a) are indicated. Amino acids matching Ecc protease are boxed. Abbreviations are: BT, B. thermoproteolyticus thermolysin; BS, B. subtilis neutral protease; PA, P. aeruginosa elastase; LP, L. pneumophila metalloprotease; SM, S. marcescens neutral protease; and ECH, E. chrysanthemi metalloprotease B.

Table 1. Effects of metal ion chelators and protease inhibitors on E. carotovora subsp. carotovora protease (Prt1) activity from prt1 cloned into plasmid pSK23 and produced by E. coli strain DH5α

Inhibitor	Concentration (mM)	Remaining enzyme activity ± S.D. (%)[1]
None	None	100 ± 12.1a[2]
EDTA	1	99 ± 0.5a
	10	91 ± 1.6a
	50	91 ± 7.4a
Phenanthroline	1	22 ± 0.9 b
	10	12 ± 11.0 b
	50	0 ± 0.0 b
Phosphoramidon	1	76 ± 3.9 b
	5	47 ± 4.0 b
PMSF	1	100 ± 9.4a
	10	100 ± 12.3a
3,4-DCI	1	100 ± 4.7a
Iodoacetate	1	100 ± 13.3a
Pepstatin	0.1	95 ± 9.7a

[1] Incubation of protease with inhibitors and remaining protease activity was measured as described in Materials and Methods. Each treatment was replicated twice.

[2] Means followed by a different letter were significantly different at the $P \leq 0.01$ level as determined by Duncan's multiple range test.

plasmid, pSK16,were Amp^r, Kan^r, Prt^-, and Tet^s. Plasmid pSK16 was mobilized into Ecc by triparental mating and marker exchange mutagenesis of Ecc was forced in phosphate-limiting medium. Homologous recombinants between prt1::kan of pSK16 and wildtype prt1 in the Ecc genome were selected by screening the bacteria for Amp^s, Kan^r, and $Prt1^-$.

Several Kan^r mutants of Ecc were detected that had reduced protease activity on gelatin plates. One, designated as L-957, was selected for Southern analysis, which indicated that kan was inserted into prt1 causing a 1.2 kb gain in mass for a 6.5 kb fragment from an EcoRI Ecc chromosomal DNA digest (Fig. 8). Protease assay of the mutant on gelatin plates showed that protease activity was approximately 60 to 80% reduced compared to the wildtype (Fig. 9). Weak hybridization occurred between the probe for prt1 and a 4.4 kb EcoRI fragment in the mutant and possibly in wildtype Ecc. This may represent a second protease gene and may account for the protease activity remaining in mutant L-957.

DISCUSSION

Little is known about the characteristics or role(s) of proteases in soft rot caused by erwinias. Here we describe the nucleotide sequence of the prt1 encoding a protease (Prt1) from Ecc strain EC14, partial characterization of Prt1, and construction of a Prt1-deficient mutant of Ecc.

The deduced amino acid sequence of prt1 showed a high degree of

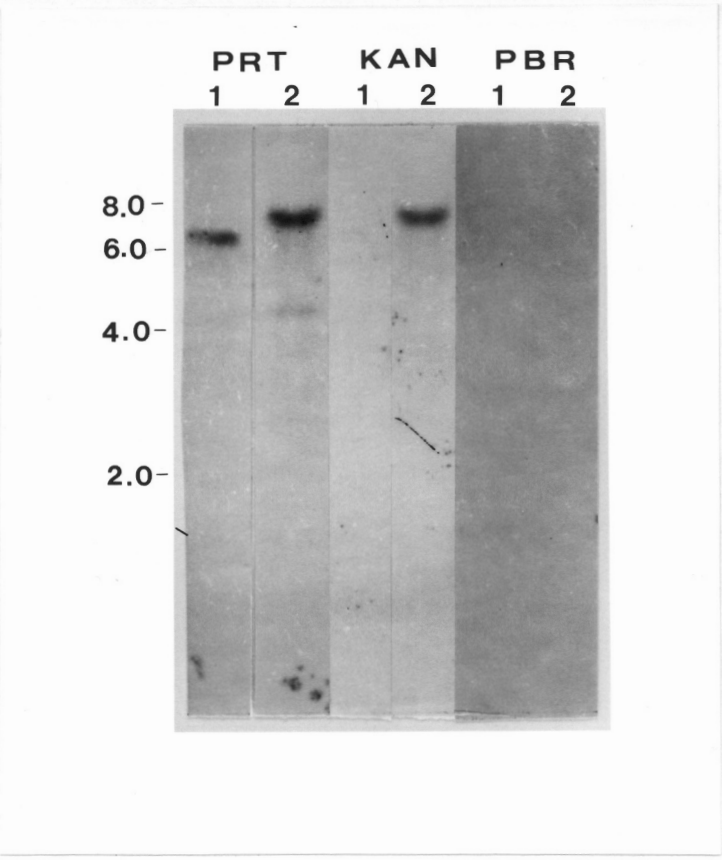


Fig. 8. Southern analyses of E. carotovora subsp. carotovora (lane 1) and prt1 site replacement mutant L-957 (lane 2). The probes (prt, kan, and pBR322) used for hybridization are indicated on the top and DNA molecular size markers (in kb) are on the left. A 1.0 kb SmaI-EcoRI fragment from pSK1 (Fig. 1) was used as the prt1 probe (PRT). A 1.2 kb SmaI fragment from pUC4-KIXX was a probe for the kanamycin resistance gene (KAN). EcoRI digested pBR322 (4.4 kb) was used as the probe for the vector (PBR). In control digestions (data not shown), PRT hybridized with 4.4 kb EcoRI linearized pSK23 (Fig.1), KAN only hybridized to a 1.2 kb pUC4-KIXX, and PBR hybridized with the 4.4 kb EcoRI-linearized pSK23, 4.4 kb EcoRI-linearized pBR322, and 2.8 kb SmaI-fragment of pUC4-KIXX.

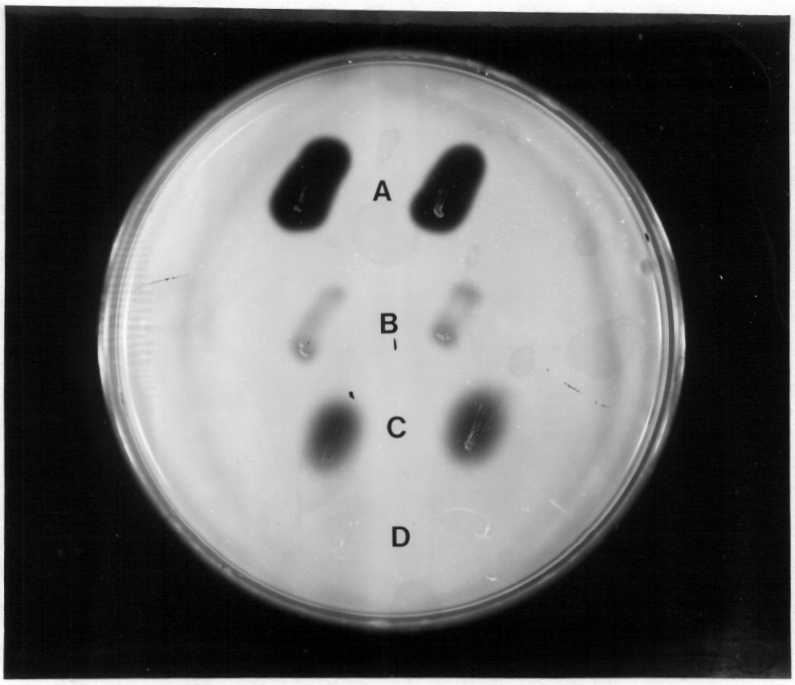


Fig. 9. Assay of protease activity of E. carotovora subsp. carotovora (A), prt1 site replacement mutant L-957 (B), E. coli/pSK23 (C), and E. coli (D) on gelatin plates. Bacteria were grown on plate at 30°C for 24 h, after which gelatin was precipitated by flooding the plates with $HgCl_2$. Clear zones in the white precipitate indicate areas of protease activity.

sequence identity to several bacterial metalloproteases. This is consistent with the inhibition of the cloned protease by phenanthroline, an inhibitor of Zn-metalloproteases with a high chelation affinity for zinc. The derived amino acid sequence of prt1 is more similar to the thermolysin from B. thermoproteolyticus than to the metalloproteases from S. marcescens (Nakahama et al., 1986). Similarity to thermolysin was also suggested by the inhibition of Prt1 activity by phosphoramidon, a zinc-ion chelator that binds specifically to the thermolysin active site. In contrast, all identified extracellular metalloproteases from E. chrysanthemi, a related soft rot pathogen, have higher sequence identity to the S. marcescens proteases. This report is the first to characterize a metalloprotease from E. carotovora.

Amino acid sequence comparison of Prt1 with thermolysin (Matthews et al., 1972a; 1972b) shows that all six active site residues, all three zinc binding residues, and five of seven substrate binding residues are identical (Table 2). Similar conservation of these sites has been described for other thermolysin-type proteases (Bever and Iglewski, 1988; Fukushima et al., 1989; Nakahama et al., 1986; Yang et al., 1984). In the predicted substrate binding site, the two amino acid changes, from residues Leu-133 and Val-192 in thermolysin to Phe-153 and Leu-216 in Prt1, respectively, represent conservative changes and do not alter hydrophobicity. A helix (residues 137 to 150) connecting the two domains of the peptide and a second helix (residues 160 to 180) lining the hydrophobic substrate binding pocket of thermolysin (Matthews et al., 1972a) are conserved in Prt1 (residues 156 to 170 and 180 to 200,

Table 2. Comparison of amino acid residues from E. carotovora subsp. carotovora (Ecc) protease with the residues implicated in B. thermoproteolyticus thermolysin function

Thermolysin	Ecc
Active site:	
Glu-143	Glu-163
Tyr-157	Tyr-177
Asp-170	Asp-190
Arg-203	Arg-227
Asp-226	Asp-259
His-231	His-264
Zinc binding sites:	
His-142	His-162
His-146	His-166
Glu-166	Glu-186
Substrate binding site:	
Phe-130	Phe-150
Leu-133	Phe-153
Val-139	Val-159
Ile-188	Ile-212
Gly-189	Gly-213
Val-192	Leu-216
Leu-202	Leu-226

respectively). The thermolysin calcium-binding sites were not conserved in Prt1, which may suggest that Prt1 does not require calcium for its activity. This is also supported by the slight inhibition of Prt1 activity by EDTA, a chelator with high affinity for calcium.

The high degree of amino acid identity between Prt1 and thermolysin in the substrate binding site suggests similarities in the substrate specificity. Thermolysin has been shown to cleave plant proteins such as the heme-free horseradish (Armoracia rusticana Gaertn. et al.) (Welinder and Smillie, 1971) peroxidase and hydroxyproline-rich glycoproteins (HRGPs) (Adair and Appel, 1989). HRGPs are located in the plant cell wall and serve a structural and defense function against microbial attack (Showalter et al., 1985). Being highly positively charged molecules, they may interact strongly with negatively charged Prt1. Collagen, a major structural unit in animal connective tissue, like HRGPs, is hydroxyproline-rich and is cleaved by thermolysin-like metalloproteases from P. aeruginosa and L. pneumophila (Conlan et al., 1986; Heck et al., 1986). Degradation of potato cell wall HRGPs (soluble extensins) by an Ecc extracellular protease has been demonstrated (Lewosc et al., 1989).

The NH_2-terminus of the deduced polypeptide from the prt1 gene shows a typical E. coli signal sequence (Oliver, 1985). This 20 amino acid sequence has two positively charged amino acids (lysine and arginine), followed by a hydrophobic core of 12 amino acids as indicated by the hydropathy plot generated by Kyte-Doolittle algorithm (Kyte and Doolittle, 1982) and a putative isoleucine-isoleucine-alanine signal

cleavage site. Signal sequences have been identified in several other extracellular metalloproteases (Bever and Iglewski, 1988; Nakahama et al., 1986; Takagi et al., 1985; Vasantha et al., 1984; Yang et al., 1984). In the E. chrysanthemi metalloprotease B sequence, a short (16 residues) NH_2-terminal pro sequence was found, but no signal sequence was observed (Delepelaire and Wandersman, 1989). The COOH-terminal region of Ecc protease contains mainly polar and charged residues (60%), and may be α-helical as indicated by a hydropathy plot. COOH-terminal processing involved in the secretion of proteases has been reported (Miyazaki et al., 1989; Pohlner et al., 1987). We have not confirmed processing.

Partial loss of detectable protease activity by the marker exchange mutant on gelatin plates suggests that at least two proteases may be produced by Ecc. Our preliminary analysis of extracellular proteins of Ecc grown in PEM and run on SDS-PAGE showed the presence of a 45 kDa protease, but not a 38 kDa protease as predicted by the prt1 gene. The absence of the 38 kDa protease in Ecc grown in rich broth could suggest that Prt1 is not induced under these conditions, but is induced by gelatin or in planta (Chapter II). The 45 kDa protease is close to the size previously reported for an extracellular protease in Ecc strain EC14 (Smith et al., 1987). We are currently characterizing this second Ecc protease (Prt2) and comparing it to Prt1, the 38 kDa protease.

Only one protease (pI 4.6 to 4.8) has been described from Ecc strains 71 and SR394 (A. Kelman, North Carolina State University,

personal communication; Willis and Chatterjee, 1987). In E. chrysanthemi, one to three extracellular proteases are produced per strain. These have been identified as metallo- (3), serine (1), or unknown proteases and range in mass from 50 to 55 kDa and in pI from 4.6 to 5.8 (Barras et al., 1986; Delepelaire and Wandersman, 1989; Wandersman et al., 1986; 1987). Differences in the number and type of secreted proteases may vary in plant pathogenic erwinias similar to the variations observed for pectolytic enzymes (Kotoujansky, 1987).

The role(s) of bacterial metalloproteases in plant or animal pathogens remains unclear. Several metalloproteases with characteristics similar to Prt1 have been reported in human pathogens (Bever and Iglewski, 1988; Black et al., 1990; Booth et al., 1983; Dreyfus and Iglewski, 1986; Fukushima et al., 1989; McKevitt et al., 1989; Morihara and Homma, 1985). In these pathogens, no single factor has been identified as sufficient for the production of all disease symptoms. Secreted proteases may enhance virulence by releasing nutrients from the host and/or by degrading host defense proteins. So far, we do not know the significance of Prt1 in soft rot. However, the detection of elevated levels of prt1 mRNA from in planta-grown Ecc indicates that this protease is produced during potato maceration. Studies with the prt1::kan marker exchange mutant, L-957, will be helpful in elucidating the role of this protease in plant pathogenesis.

ACKNOWLEDGMENTS

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III. ERWINIA CAROTOVORA SUBSP. CAROTOVORA EXTRACELLULAR PROTEASES: ROLE IN PATHOGENESIS

To be submitted to **Phytopathology** or **Molecular Plant-Microbe Interactions** by S.M.R. Kyöstiö, C.L. Cramer, and G.H. Lacy

ABSTRACT

An extracellular protease (Prt2) in Erwinia carotovora subsp. carotovora (Ecc) was characterized and differs from the previously characterized 38 kDa Ecc protease (Prt1). Prt2 has a molecular mass of 45 kDa, pI of 4.8, and pH and temperature optima of 7.5 and 40°C, respectively. The NH_2-terminal amino acid sequence of Prt2 shows significant sequence identity with the NH_2-termini of Erwinia chrysanthemi and Serratia marcescens metalloproteases, but not to Prt1. The role of proteases in potato tuber maceration was assessed using a marker exchange mutant of prt1 and a protease-deficient ($Prt1^-$, $Prt2^-$) Tn5 mutant of Ecc. The mutants were reduced 20 to 30% in maceration compared to the wildtype Ecc. Reduced maceration correlated with slower mutant growth in planta, suggesting a nutritional role for the Ecc proteases. Northern analysis of in planta grown Ecc further indicated prt1 mRNA accumulation 12 h after inoculation.

PROTEASE 2 AND PATHOGENESIS

INTRODUCTION

The soft rot pathogen, Erwinia carotovora subsp. carotovora (Jones) Bergey et al. (Ecc) strain EC14 secretes several potential plant cell-degrading enzymes (Roberts et al., 1986; Stack et al., 1980; Tseng and Mount, 1973). Pectate lyases are apparently important in pathogenesis (Collmer and Keen, 1986; Kotoujansky, 1987). However, little is known about the possible roles for the other enzymes in pathogenesis. Bacterial protease(s) have a potential role in degrading proteinaceous structural components of plant cell walls such as extensins and membrane proteins, cleaving defense proteins such as chitinases or protease inhibitors, or providing substrates for bacterial nutrition.

The role of proteases from plant pathogens in pathogenesis has been studied mostly as a nutritional benefit that may aid bacteria in growth and colonization of the host. This is supported by decreased growth rates in planta of protease-deficient mutants. A protease-deficient Xanthomonas campestris pv. malvacearum (Smith) Dye grew two- to three-fold slower in planta than the wildtype (Gholson, et al., 1989). A Tn4431-induced protease-deficient mutant of Xanthomonas campestris pv. oryzae (Ishiyama) Dye had 10- to 100-fold lower populations in planta and was reduced in virulence (Xu and Gonzales, 1989). However, a Tn5-induced serine protease mutant of Xanthomonas campestris pv. campestris (Pammel) Dowson had a similar growth rate and final population size compared to the wildtype (Liu et al., 1990; Tang et al., 1987).

Previously, we identified a 38 kDa Ecc protease (Prt1), which is produced during growth in planta (Chapter II). Prt1 is similar to extracellular metalloproteases from Pseudomonas aeruginosa (Schoeter) Migula, Legionella pneumophila Brenner et al., and Vibrio cholerae Pacini (reviewed in Chapter II), which are involved in several diseases of animals. These proteases facilitate host tissue colonization by degrading structural components, evading host defenses, and making host proteins available for bacterial nutrition and growth (Bever and Iglewski, 1988; Black et al., 1990; Booth et al., 1983).

Marker exchange mutagenesis of the Ecc prt1 gene caused a reduction in protease production, but did not completely abolish it (Chapter II), suggesting the presence of a second protease. Further, protease detection on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed a 45 kDa extracellular protease (Prt2) differing from Prt1 (38 kDa). The mass of Prt2 resembles previously identified Ecc extracellular proteases. Smith et al. (1987) reported a 50 kDa protease with a pI of 4.8 from the same Ecc strain. Tseng and Mount (1973), also studying EC14, reported a protease with a pI of 8.3, but its molecular mass was not determined. J. Lewosc, A. Kelman, and L. Sequeira (University of Wisconsin, Madison and North Carolina State University, personal communications) studied a 43 kDa metalloprotease in Ecc strain SR394. This protease digests soluble but not insoluble extensins extracted from plant cell walls. This suggests a role for that protease in pathogenesis; it may reduce cell wall integrity and slow conversion of soluble to insoluble extensins thus possibly

interfering at some level with host defense responses (Lamb et al., 1990).

The objectives of this paper were (1) to characterize the 45 kDa Ecc protease (Prt2) and compare it to the 38 kDa protease (Prt1), (2) to elucidate the overall effect of proteases in soft rot by measuring the maceration ability of various Ecc protease-deficient mutants on potato tuber slices, and (3) to compare the kinetics of protease expression during pathogenesis to that of other cell-degrading enzymes.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and chemicals

Erwinia carotovora subsp. carotovora (Ecc) and Escherichia coli (Migula) Castellani and Chalmers strains used here are described in Table 1 and Fig. 1. Plasmids pSK1 and pSK23 have been described previously (Chapter II). Bacteria were cultured on LB broth (Difco, Detroit, Mi.), LB agar (Difco), Kings medium B (KB; King et al., 1954), mineral medium (Zucker and Hankin, 1970) with 1% glycerol, protein extraction medium (PEM; Chapter II), or PEC-YA plates (Starr et al., 1977) containing appropriate antibiotics (50 µg/ml ampicillin or 25 µg/ml kanamycin). Gelatin plates (Hankin and Anagnostakis, 1975) were used to detect protease activity. Antibiotics and chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise stated.

Partial purification and characterization of Prt2

Ecc was grown with orbital shaking (100 rpm) in two liters of PEM

Table 1. Bacterial strains used in this study

Strain[a]	Relevant genotype or phenotype	Reference
	E. carotovora subsp. *carotovora* (Ecc) strains:	
EC14	Wildtype pathogen[b], pectolytic (Pec^+)[c], $Prt1^+$, $Prt2^+$, Amp^s, Kan^s	Mount et al., 1979
L-763	Protease-deficient[d] Tn*5* mutant of EC14, Pec^+, $Prt1^-$, $Prt2^-$, Amp^s, Kan^r	Allen et al., 1986
L-957	Marker-exchange mutant of *prt*1 in EC14, Pec^+, $Prt1^-$, $Prt2^+$, Amp^s, Kan^r	Chapter II
	E. coli strain:	
DH5α/pSK23	Transformed with plasmid pSK23 containing *prt*1 in a 1.8 kb *Nru*I fragment, $Prt1^+$, Amp^r, Kan^s	Chapter II

[a] Strain names with the prefix L-, collection of G.H. Lacy, Virginia Polytechnic Institute and State University, Blacksburg, Va., USA.
[b] Isolated from yellow calla lily (*Calla palustris* L.) by A.P. Ark in California and provided by R.S. Dickey, retired, Cornell University, Ithaca, N.Y. USA.
[c] Pectolytic activity determined by the method of Starr et al. (1977).
[d] Protease activity determined by gelatin plate assay. See Materials and Methods and Fig. 1.

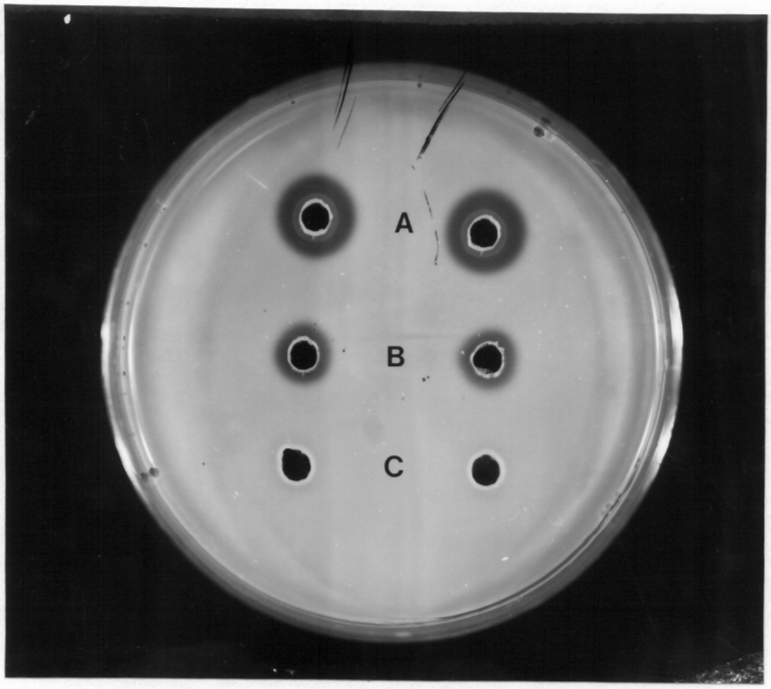


Fig. 1. Protease activity of (A) E. carotovora subsp. carotovora strain EC14 and its mutants (B) L-957, a prt1 site replacement mutant, and (C) L-763, a Tn5 transposon mutant (see Table 1). Bacteria were grown at 30°C in LB broth to turbidity at 600 nm of 0.8 and 25 µl of the culture was added to wells in gelatin plates (See Materials and Methods). After overnight incubation at 30°C the plates were flooded with $HgCl_2$ and protease activity was detected as a clear zone in the white gelatin precipitate.

at 30°C overnight. The culture was centrifuged at 4°C and 4,000 x g for 15 min and the supernatant saved. Extracellular proteins were precipitated from the supernatant by adding ammonium sulfate to 80% saturation and incubating for 3 h at 4°C. Precipitated proteins were pelleted by centrifugation at 20,000 x g for 30 min at 4°C. The pellets were resuspended in 10 ml of 10 mM Tris-HCl (pH 8.0) containing 2 mM $CaCl_2$ and dialyzed against the same buffer overnight. Calcium was added to all buffers during concentration and enzyme assays, since it stabilizes bacterial metalloproteases (Barrett, 1986). The dialysate was concentrated five-fold by ultrafiltration (exclusion limit 10 kDa; Centriprep-10, Amicon, Danvers, Ma.) and loaded onto a Mono Q HR 5/5 anion exchange column (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) equilibrated with 10 mM Tris-HCl (pH 8.0) containing 2 mM $CaCl_2$. Proteins were eluted with a linear gradient of 0.0 to 1.0 M NaCl. Column fractions (1 ml) were measured for protease activity using azocasein as substrate (described in Chapter II) and protein concentration by the method of Bradford (1976). One unit (U) of protease activity was defined by the azocasein method as an absorbance ($A_{340\ nm}$) increase of 0.001 min^{-1}. Fractions containing protease activity were concentrated five-fold by ultrafiltration (Centricon-10 microconcentrators; exclusion limit 10 kDa; Amicon), applied to a gel filtration column (Superose 12 HR 10/30; Pharmacia) equilibrated with 10 mM Tris-HCl (pH 8.0) containing 2 mM $CaCl_2$, and eluted with the same buffer. In subsequent purifications, gel filtration was omitted since no additional separation of proteins was obtained by this procedure.

The molecular mass of Ecc extracellular protease was determined by 12% (w/v) SDS-PAGE using proteins (25 μg) boiled for 5 min in loading buffer (Laemmli, 1970). The protease band was identified using a gelatin overlay as described previously (Chapter II). Proteins were stained with Coomassie blue (Schleif and Wensink, 1981). Protein mass was estimated by comparison with mass standards (High molecular weight standards, BRL; Bethesda Research Laboratories).

Isoelectric focusing was performed as previously described (Chapter II).

The effect of any protease-inhibiting Ecc proteins was assayed by adding Ecc intracellular proteins to Prt1 and Prt2 preparations. Prt1 was obtained from intracellular proteins from toluene-lysed E. coli/pSK23 cells (5 ml LB overnight culture). Prt2 was obtained by concentrating the supernatant from PEM-grown Ecc cells by ammonium sulfate precipitation as described above. Ecc intracellular proteins were prepared by lysing a resuspended cell pellet (1 g cells/1 ml 0.1 M Tris-HCl [pH 8.0]) from 1 liter of PEM-grown Ecc by sonicating six times for 30 sec at setting 3 (Sonicator™, Heat Systems-Ultrasonics, Inc., model W-220F). The cells were cooled in an ice bath (0°C) after each sonication to prevent temperature elevation. Proteins were precipitated with 0 to 50% or 50 to 95% ammonium sulfate. The precipitate was centrifuged at 4°C and 10,000 x g for 30 min, and dialyzed against 0.1 M Tris-HCl (pH 8.0). Intracellular proteins (0 to 600 mg) were preincubated with the protease at 30°C for 20 min and any remaining protease activity was measured using azocasein as substrate as described

previously (Chapter II).

The effect of metal ion chelators, EDTA (ethylenediaminetetra-acetic acid) and phenanthroline, and protease inhibitors, 3,4-dichloro-isocoumarin (3,4-DCI), iodoacetate, pepstatin, phenylmethanesulfonyl fluoride (PMSF), and phosphoramidon on Ecc Prt2 activity was measured as described in Chapter II.

The pH and temperature optima of the protease were determined using azocasein as substrate as previously described (Chapter II). In pH studies, protease activity in concentrated extracellular supernatant was assayed in the following 0.1 M buffers: sodium-acetate (pH 4.5 to 5.5), sodium-phosphate (pH 6.0 to 7.0), Tris-HCl (pH 7.5 to 8.5), and glycine-NaOH (9.0 to 10.0). The effect of temperature was observed from 20 to 65°C at pH 8.0.

To test the effect of cations on protease activity, concentrated Ecc culture supernatant from ammonium sulfate precipitation (as described above) was preincubated with 1 mM EDTA to chelate endogenous cations at 30°C for 20 min (Roberts et al., 1986; Stack et al., 1980). Various cations (10 mM $CaCl_2$, $ZnCl_2$, $MgCl_2$, $CoCl_2$, or $CuCl_2$) were added to the sample and incubated further under same conditions for 20 min to test their ability to restore protease activity on azocasein.

Amino acid composition and NH_2-terminal sequencing for Prt2

For these analyses, partially purified Prt2 was separated on 12% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Matsudaira, 1987), stained with Coomassie Blue, and the Prt2 band (45 kDa) identified by gelatin overlay was excised. Amino acid composition

(Cohen et al., 1984) and NH_2- terminal sequence (Niall, 1977) were determined by Dr. S. Kyin at the University of Illinois Biotechnology Center, Urbana, Il.

RNA isolation and Northern analysis

In planta inoculation using a membrane-separated system, total bacterial RNA isolation, and Northern hybridizations were performed as previously described (Yang et al., 1989). A 1.0 kb SmaI-EcoRI fragment of pSK1 was used as a probe for prt1 mRNA.

Maceration assay

The maceration assays were performed at 30°C for 24 h by the method of Keen et al. (1984) as described in Roberts et al. (1986). Wells (5 mm dia.) cut with a #2 corkborer in potato (Solanum tuberosum L. cv. Russet Burbank) tuber slices were inoculated with 50 µl of bacterial culture (2 X 10^8 cfu/ml) using bacteria grown to the late exponential phase (turbidity at 600 nm = 0.8) in LB at 30°C with orbital shaking (100 rpm). Assays were carried out in humidified plastic chambers (Yang et al., 1989) or anaerobic jars with CO_2/H_2 generators (GasPak Anaerobic System; Baltimore Biological Laboratories, Cockeysville, Md.) as described by Maher and Kelman (1983). Maceration was measured by loss of tissue weight (Yang et al., 1989).

In planta growth measurements

Bacteria were cultured in mineral media supplemented with glycerol at 30°C with orbital shaking (100 rpm) until a turbidity of 0.8 at 600 nm was reached. Potato tuber slices were prepared as described above except that no wells were cut in the surface. Instead, the surface of

each slice was covered with a polysulfone membrane (Yang et al., 1989; GA-8S, 0.2µm pore size; Gelman Sciences, Ann Arbor, Mi.) on which the bacterial inoculum (0.3 to 1.2 x 10^8 cfu in 400 µl of the 1% glycerol mineral medium) was spread. The inoculated tuber slices were incubated in humidified plastic chambers. At time 0 and after 6, 12, 24, or 48 h of incubation, bacteria were washed off the membrane and plated on KB with or without kanamycin as appropriate to the strain (Table 1). After overnight incubation at 30°C, the non-fluorescent Ecc colonies were inoculated onto PEC-YA medium and incubated as described above. To visualize pectate lyase production, the PEC-YA plates were flooded with 2 N HCl to precipitate remaining sodium polypectate (Starr et al., 1977).

Growth rate measurements

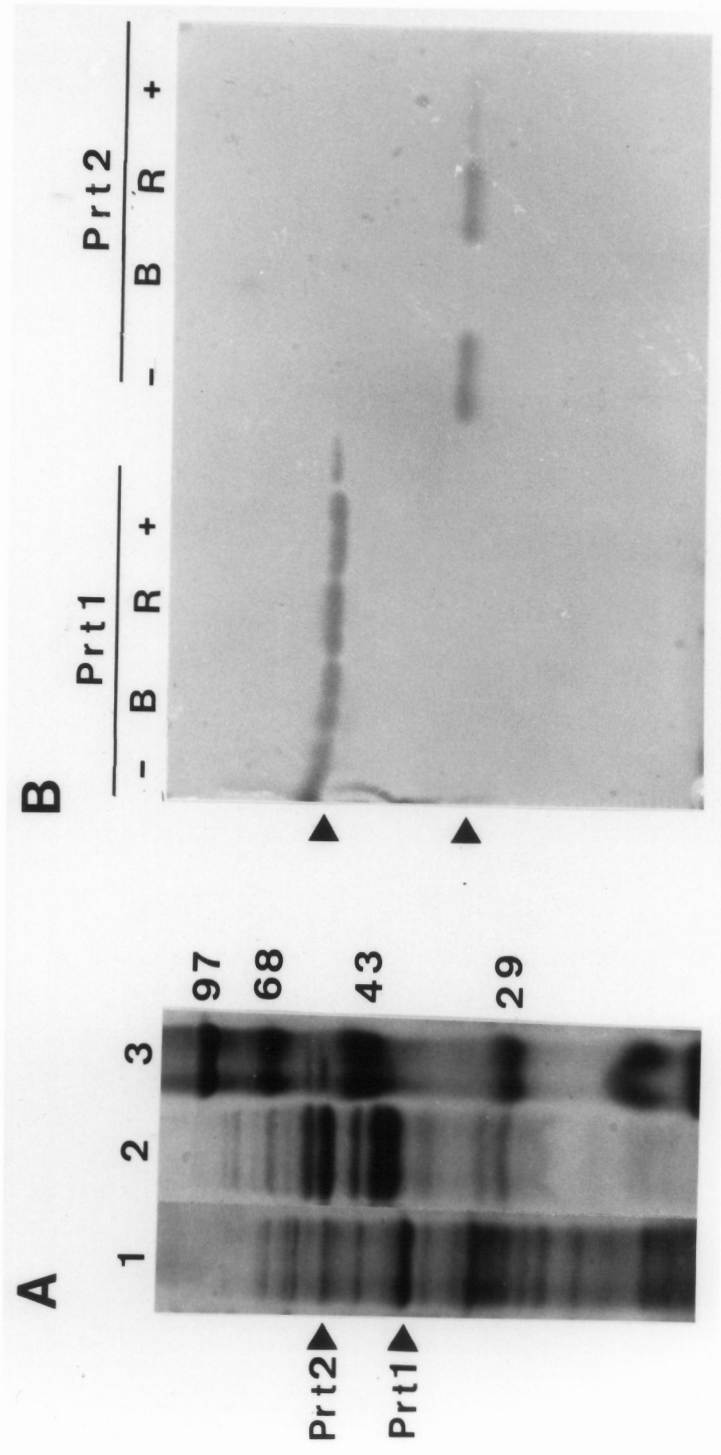
Overnight LB cultures were diluted 1:100 into 100 ml of mineral medium plus 1% glycerol or 100 ml of LB broth containing appropriate antibiotics. Growth was followed by measuring turbidity at 600 nm. Growth rates in the mid-exponential growth phase were estimated by measuring the time required for the turbidity to double.

RESULTS

Characterization of the Prt2

A single protease (Prt2) capable of digesting gelatin was detected in the extracellular protein fraction of Ecc grown in PEM (Fig. 2). The molecular mass of Prt2, estimated to be 45 kDa by 12% SDS-PAGE and gelatin overlay, differs from the 38 kDa Prt1 (Fig. 2A). Prt2 was heat-

Fig. 2. Comparison of molecular masses of E. carotovora subsp. carotovora Prt1 and Prt2 by 12% SDS-PAGE. A. Lane 1, Prt1 from prt1 expressed intracellularly in E. coli DH5α/pSK23; lane 2, Prt2 from extracellular protein fraction of Ecc grown in PEM; lane 3, molecular mass standards (kDa). B. Protease activity assay by gelatin overlay of SDS-PAGE using unboiled and nonreduced (-), boiled for 5 min (B), reduced with ß-mercaptoethanol (R), or boiled and reduced samples (+) of Prt2 or Prt1.


A
1
2
3
97
68
43
29
Prt2
Prt1
B
Prt1
- B R +
Prt2
- B R +

stabile, retaining enzyme activity after boiling, while Prt1 lost enzyme activity after boiling (Fig. 2B). Since no Prt2 activity was detected in the Ecc intracellular fraction, the effect of Ecc intracellular proteins on Prt2 activity was tested; Prt2 was inhibited 96% by 0.6 mg of Ecc intracellular proteins, while the same amount of intracellular proteins did not reduce Prt1 activity significantly (Table 2). Prt2 activity in a concentrated culture supernatant of PEM-grown Ecc was inhibited by EDTA and 1,10-phenanthroline, but not by PMSF, 3,4-DCI, iodoacetate, or pepstatin (Table 3), suggesting that it is a metalloprotease. Prt2 was not inhibited by 1.0 mM phosphoramidon, indicating that this protease is not a Bacillus thermoproteolyticus Rokko thermolysin-like metalloprotease in contrast to Prt1 (Chapter II). Prt2 had activity over a broad pH range with its optimum between pH 7.5 to 8.0 (Appendix D). The temperature optimum for Prt2 was 40°C, although 46% of its activity was retained at 60°C (Appendix D). Among divalent cations tested, only $CaCl_2$ restored 100% of the Prt2 activity after chelation of endogenous ions with 1 mM EDTA (Appendix D). In contrast, Prt1 was not inhibited greatly by EDTA suggesting that calcium is not required for its activity (Chapter II).

The first 20 amino acids of Prt2 determined by NH_2-terminal sequencing were 55 and 60% identical to the NH_2-terminal sequence of Serratia marcescens Bizio metalloprotease (Nakahama et al., 1986) and Erwinia chrysanthemi Burkholder et al. protease B (Delepelaire and Wandersman, 1989), respectively (Fig. 3). All these proteases had a conserved sequence of TGY-XX-VXD-XXX-YH-X-RG in the NH_2-terminus. No

Table 2. Effect of E. carotovora subsp. carotovora (Ecc) intracellular proteins on Prt1 and Prt2 activities

Fraction tested[1]	Ecc intracellular proteins[1] (mg)	Activity[2] (%)
Incubated with Prt1 (E. coli/pSK23 intracellular proteins)[1]		
Ecc intracellular proteins:		
50-95% ammonium sulfate fraction (unboiled)	0.0	100.0 ± 2.1a[3]
	0.12	97.7 ± 2.1ab
	0.30	92.0 ± 4.2abc
	0.60	88.0 ± 3.5 bcd
Incubated with Prt2 (Ecc extracellular proteins)[1]		
Ecc intracellular proteins:		
0-50% ammonium sulfate fraction (unboiled)	0.00	100.0 ± 1.8abc
	0.60	99.7 ± 0.8ab
50-95% ammonium sulfate fraction (unboiled)	0.00	100.0 ± 0.1ab
	0.12	84.8 ± 1.6 cd
	0.30	30.3 ± 4.4 e
	0.60	4.0 ± 0.6 f
50-95% fraction (boiled[4])	0.00	100.0 ± 2.2ab
	0.12	80.4 ± 2.4a
	0.30	25.5 ± 4.8 e
	0.60	4.0 ± 0.7 f

[1] Ecc intracellular and extracellular proteins, and E. coli/pSK23 intracellular proteins were prepared as described in Materials and Methods.

[2] Remaining protease activity after preincubation with intracellular proteins was assayed using azocasein as substrate as described in Chapter II. Protease activity is described as percentage of the control activity with no intracellular proteins. Numbers are means of two determinations.

[3] Means followed by a different letter were significantly different at the $P \leq 0.01$ level as determined by Duncan's multiple range test.

[4] The heat stability of the inhibiting factor was studied by boiling intracellular proteins for 5 min before adding to the protease fraction.

Table 3. Effects of metal ion chelators and protease inhibitors on E. carotovora subsp. carotovora (Ecc) extracellular protease (Prt2) activity

Effector	Concentration (mM)	Remaining enzyme activity (%)[1]
None		100.0 ± 2.6ab[2]
EDTA	1	88.0 ± 5.5 b
	10	19.4 ± 1.2 e
Phenanthroline	1	35.6 ± 9.3 e
	10	10.3 ± 6.0 f
Phosphoramidon	1	100.0 ± 5.5a
PMSF	1	84.5 ± 5.2 c
	10	74.6 ± 11.6 d
3,4-DCI	1	91.0 ± 9.1 b
Iodoacetate	1	93.3 ± 3.8 b
Pepstatin	1	98.9 ± 1.3 b

[1] Ecc protease activity was determined using azocasein as substrate. Approximately 25 U of protease concentrated from culture supernatant was preincubated with inhibitors at 30°C for 20 min before protease assay. Values represent the mean ± standard deviation from six determinations.

[2] Means followed by a different letter were significantly different at the $P \leq 0.01$ level as determined by Duncan's multiple range test.

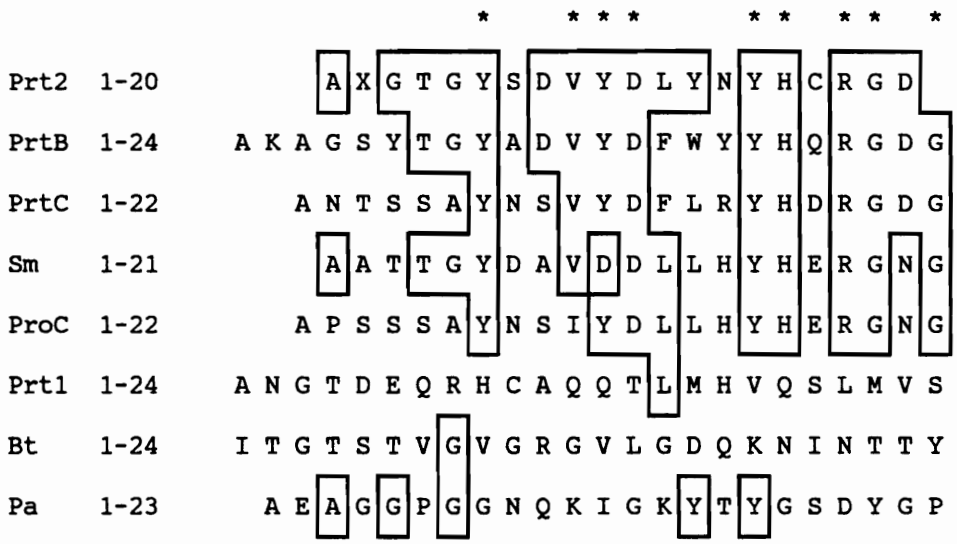


Fig. 3. Comparison of the NH_2-terminal amino acid sequence of purified E. carotovora subsp. carotovora protease (Prt2) to other bacterial metalloproteases. Numbers refer to the location of the residues in the amino acid sequence for each mature protease. Conserved residues are indicated by asterisks (*). Other abbreviations are: PrtB, E. chrysanthemi protease B; PrtC, E. chrysanthemi protease C; Sm, S. marcescens neutral protease; ProC, E. chrysanthemi EC16 protease C;, Prt1, E. carotovora subsp. carotovora prt1 (residues 20 to 41 from the deduced polypeptide, putative signal sequence starts from residue 20), B. thermoproteolyticus thermolysin (Bt), and P. aeruginosa elastase (Pa).

significant similarity of the Prt2 NH_2-terminal sequence was found to Ecc Prt1 (deduced amino acid sequence; Chapter II), B. thermoproteolyticus thermolysin (Titani et al., 1972), or P. aeruginosa elastase (Schad et al., 1987). The amino acid composition of Ecc Prt2 is compared to E. chrysanthemi, S. marcescens, B. thermoproteolyticus proteases, and the deduced composition of Ecc Prt1 in Table 4.

Virulence of Ecc protease-deficient mutants

To determine if Prt1 or Prt2 play a significant role in pathogenesis, virulence, the amount of maceration caused by the wildtype pathogen and the protease mutants was compared. Marker exchange (L-957) and transposon (L-763) mutants of Ecc were reduced consistently and significantly ($P \leq 0.01$) compared to the wildtype in their ability to macerate potato tuber slices after 24 h incubation both in aerobic and anaerobic conditions (Table 5). This maceration assay was performed with direct contact between the bacteria and the potato tuber tissue and, thus, differs slightly in kinetics from the maceration assay using membrane-separated system shown later.

Growth of Ecc and protease-deficient mutants

To determine if the reduced maceration by the mutants was due to slower growth in planta compared to wildtype Ecc, the growth was measured on potato tuber slices. Since bacteria are difficult to recover quantitatively from the macerated tuber tissue, we used a membrane-separated system. Bacteria were separated from the tuber tissue by a polysulfone membrane, which allowed the passege of extracellular enzymes, but not the bacteria (Yang et al., 1989). This

Table 4. Amino acid composition of E. carotovora subsp. carotovora extracellular proteases (Prt1 and Prt2) in comparison to E. chrysanthemi protease B (PrtB), S. marcescens protease (Sm), and B. thermoproteolyticus thermolysin (Bt)

	Number of residues per molecule				
Amino acid	Prt2[a]	PrtB[b]	Sm[c]	Prt1[d]	Bt[e]
Asx	65	71	73	37	44
Glx	30	35	39	46	21
Ser	25	42	35	16	26
Gly	49	53	62	31	36
His	8	12	12	18	8
Arg	14	12	11	14	10
Thr	29	43	29	16	25
Ala	37	42	49	28	28
Pro	13	11	10	12	8
Tyr	21	23	17	11	28
Val	17	24	27	18	22
Met	0	5	1	7	2
Ile	20	21	22	16	18
Leu	22	25	28	27	28
Phe	16	23	31	14	10
Lys	11	14	17	8	11
Total	377	456	463	327	316

[a] Calculated assuming mass of 45.0 kDa.
[b] Deduced from prtB resulting in mass of 51.6 kDa (Delepelaire and Wandersman, 1989).
[c] Calculated assuming mass of 50.6 kDa (Nakahama et al., 1986).
[d] Deduced from prt1 minus putative signal peptide resulting in mass of 36.5 kDa (Chapter II).
[e] Calculated assuming mass of 34.4 kDa (Titani et al., 1972).

Table 5. Maceration of potato tuber tissue by E. carotovora subsp. carotovora (Ecc) wildtype and protease-deficient mutants

Treatment	Experiment	Weight (g±SD) of macerated tissue		
		Ecc strains[1]		
		EC14	L-957	L-763
Aerobic incubation[2]	1	1.06±0.27a[3]	not tested	0.79±0.26b
	2	1.31±0.51a	0.74±0.33c	1.07±0.33b
Anaerobic incubation[4]	1	0.51±0.12d	not tested	0.25±0.05e
	2	0.56±0.25d	0.34±0.20e	0.30±0.15e

[1] EC14 = wildtype strain of Ecc; L-957 = prt1 marker exchange mutant of EC14; L-763 = transposon mutant of EC14 with no expression in vitro of prt1 and prt2.

[2] Incubated at 30°C for 24 h in a humified box.

[3] The figures reported here are the means of 10 observations in experiment 1 and 12 observations in experiment 2. Means in an experiment followed by a different letter were significantly different at the $P \leq 0.01$ level as determined by Duncan's multiple range test.

[4] Incubated as described above in an aerobic jar with CO_2/H_2 generator as described by Maher and Kelman (1983).

resulted in an efficient recovery of membrane-inoculated bacteria and decreased contamination by tuber-inhabiting indigenous bacteria. The *in planta* growth rate assay by viable count showed that the marker exchange mutant, L-957, and the Tn*5* mutant, L-763, did not differ from wildtype Ecc during exponential growth within the first 24 h of interaction (Fig. 4A). However, after 24 h *in planta*, growth of L-763 and L-957 was reduced significantly ($P \leq 0.01$) compared to wildtype Ecc (Fig. 4A). The slower growth of L-957 and L-763 correlated with reduced tuber maceration measured after removing the membrane (Fig. 4B). However, mutant L-763 was reduced less in maceration than L-957 (Fig. 4B and Table 5). Compared to the direct contact maceration assay, the membrane-separated system showed somewhat slower kinetics of maceration. In LB broth or mineral media supplemented with 1% glycerol, the growth rates of the mutants measured by turbidity at 600 nm did not differ significantly from the wildtype Ecc (Table 6).

Expression of *prt*1 during pathogenesis

Since the maceration assays with the protease mutants indicated that protease(s) play a role in tuber maceration we wanted to measure the protease production during *in planta* growth. However, because extracellular proteases move through the membrane to the tuber tissue during the interaction, protease activity is difficult to assay quantitatively. We thus monitored the protease expression at the transciptional level, which allowed us to observe whether *prt* mRNA was regulated in response to host tissue. Analysis of *prt*1 expression was studied, because *prt*1 nucleic acid probes were available and Prt1$^-$

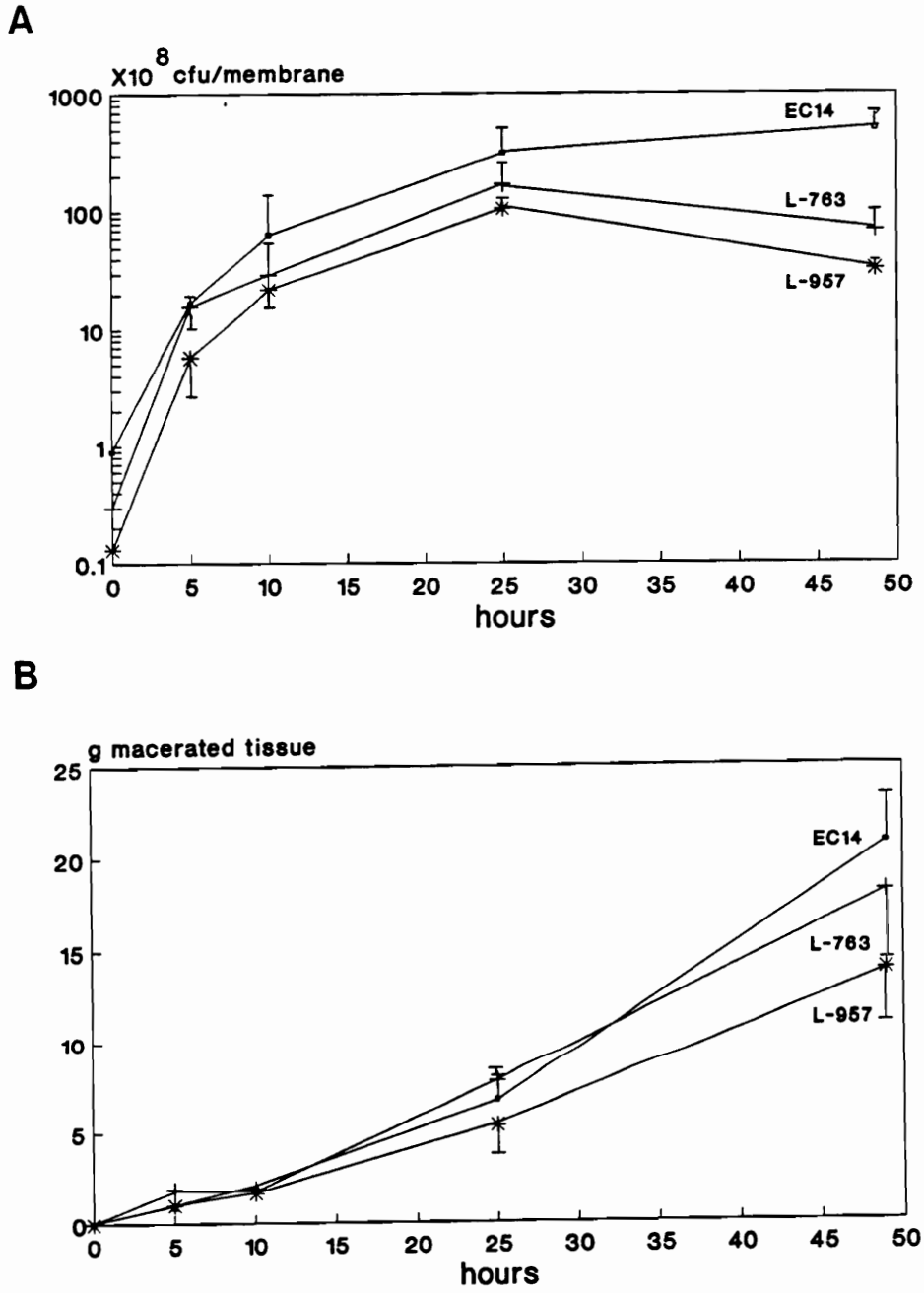


Fig. 4. In planta growth and maceration by E. carotovora subsp. carotovora wildtype (o) and its protease-deficient mutants, L-763, a Tn5 transposon mutant (+), and L-957, a prt1 site replacement mutant L-957 (*), as measured by (A) viable count on KB medium and (B) loss of weight due to maceration, respectively. Potato tuber slices were inoculated by the membrane-separated system (Yang et al., 1989) and incubated at 30°C.

Table 6. Doubling times (means) for E. carotovora subsp. carotovora (Ecc) wildtype and its protease-deficient mutants in minimal medium, rich medium, and in planta

Medium	Doubling times (h) ± S.D.		
	Ecc strain[1]		
	EC14	L-763	L-957
Mineral medium + 1% glycerol[2]	2.60 ± 0.01a[3]	2.65 ± 0.01a	2.60 ± 0.01a
LB broth[2]	1.15 ± 0.19b	1.14 ± 0.21b	1.17 ± 0.19b
in planta[4]	5.72 ± 1.00c	5.62 ± 0.50c	5.67 ± 0.17c

[1] EC14 = wildtype Ecc; L-763 = EC14 Tn5 mutant (Prt1$^-$, Prt2$^-$); L-957 = EC14 prt1 site replacement mutant (Prt1$^-$, Prt2$^+$).

[2] Bacteria were cultured in broth media at 30°C with orbital shaking (100 rpm). Doubling times were estimated by turbidity measurements at 600 nm.

[3] Means (average of three measurement) followed by a different letter were significantly different at the $P \leq 0.10$ level as determined by Duncan's multiple range test.

[4] Potato tuber slices were prepared by the method of Keen et al. (1984) as described by Roberts et al. (1986) and inoculated by the membrane-separated method of Yang et al. (1989). Cells were washed from the membrane and turbidity at 600 nm was measured. Growth rates were calculated during exponential growth.

mutant (L-957) showed similar reductions in maceration as the mutant with no protease protease production (L-763) as shown in Table 5 and Fig. 4B. Growth in planta caused an accumulation of prt1 mRNA from relatively low levels in glycerol-grown culture (time 0; Fig. 5A). Increase in the 1.3 kb transcript accumulation was first observed after 2 h. Between 2 to 4 h there was a slight increase in prt1 mRNA; at 6 h, however, accumulation decreased. This biphasic accumulation of prt1 mRNA was not observed with probes for other Ecc genes (Yang et al., 1989) suggesting that it reflects a real decrease in prt1 mRNA levels rather than variation in RNA loading. As an internal control for constitutive expression of mRNA, the membrane was also probed with a DNA fragment encoding E. coli ribosomal proteins (S5, S8, S14, S17, L18, L29; Cerretti et al., 1983) and showed no significant loading variations for the mRNAs in separate lanes. A major increase in hybridizable mRNA occured between 9 and 12 h. Accumulation of prt1 mRNA decreased by 24 h. A second band observed at 1.0 kb followed the kinetics of the 1.3 kb transcript; the 1.0 kb band may represent a processed or degraded product of the 1.3 kb transcript. The expression of prt1 resembles the transient induction kinetics for genes for Ecc pectolytic enzymes, endo-polygalacturonase (endo-PG) and endo- and exo-pectate lyase (Yang, 1990; Fig. 6B).

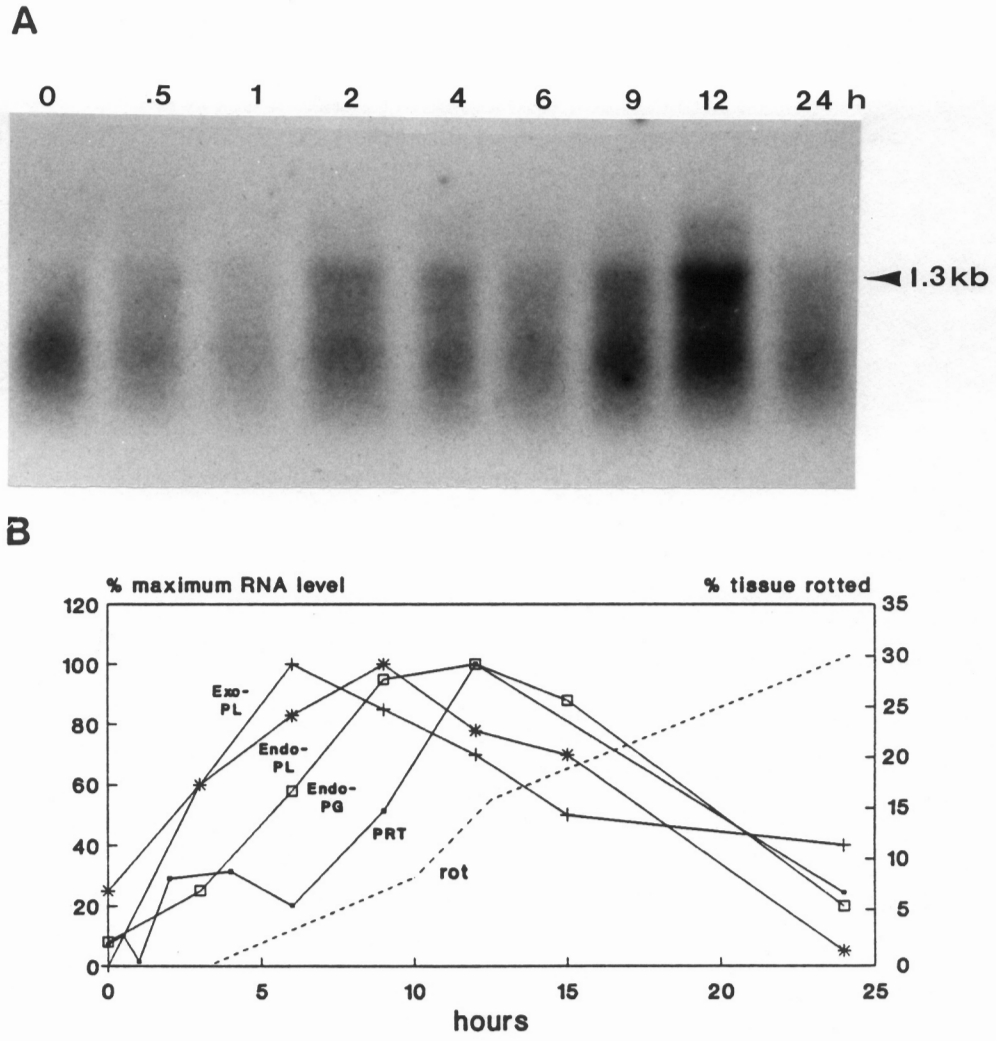


Fig. 5. Northern analysis of E. carotovora subsp. carotovora (Ecc) prt1 expression during growth on potato tuber slices. A. Bacterial RNAs were isolated at various times from Ecc induced under compatible conditions in the membrane-separated potato system, separated by electrophoresis, blotted to a membrane, and hybridized with a ^{32}P-labelled prt1 probe (1.0 kb SmaI-EcoRI fragment of pSK1). Hybridizing RNAs are indicated by the arrow and are approximately 1.3 kb in size. B. Relative mRNA accumulation levels for prt1 (-o-) presented as percent maximum density are compared with those previously observed (Yang, 1990) for in planta expression of Ecc endo-pectate lyase (endo-PL; -*-), exo-pectate lyase (exo-PL;-+-) , and endo-polygalacturonase (endo-PG; -□-). Percent tuber tissue maceration (- -) was determined by weight loss.

DISCUSSION

Our results establish that Ecc has two distinct proteases. The characteristics of these proteases, Prt1 and Prt2, are summarized in Table 7. The proteins have the same pI (4.8) and are both metalloproteases. They differ in estimated molecular mass, inhibition by both phosphoramidon and Ecc intracellular proteins, NH_2-terminal sequence, and amino acid composition.

Other plant pathogens also produce several extracellular proteases. Metalloproteases have been demonstrated in E. chrysanthemi (Delepelaire and Wandersman, 1989) and E. carotovora (Chapter II) while serine proteases have been reported in E. chrysanthemi and X. campestris pv. campestris (Liu et al., 1990; Wandersman et al., 1986). Whether the type of protease correlates with the function of the protease is unclear. Among metalloproteases, two groups of bacterial metalloproteases are becoming evident; the B. thermoproteolyticus thermolysin-type proteases and the S. marcescens metalloprotease-like proteases. So far, among plant pathogens only the Ecc 38 kDa protease (Prt1) has been identified as thermolysin-like, while this type of protease has been observed in several animal pathogens (Bever and Iglewski, 1988; Black et al., 1990; Booth et al., 1983; Dreyfus and Iglewski, 1986; Fukushima et al., 1989; McKevitt et al., 1989; Morihara and Homma, 1985).

Proteases contribute to pathogen virulence (Bever and Iglewski, 1988; Black et al., 1990; Booth et al., 1983). This has not previously been shown among soft rot plant pathogens (Dahler et al.,

Table 7. Comparison of E. carotovora subsp. carotovora proteases, Prt1 and Prt2

Characteristic	Prt1	Prt2
Molecular mass (kDa)[a]	38	45
pI[b]	4.8	4.8
Heat stability	Unstable	Stable
Type of protease	Metalloprotease	Metalloprotease
Predicted metal cofactor	Zn	Ca, Zn
Inhibition by phosphoramidon[c]	Inhibited	Not inhibited
Inhibition by Ecc intracellular proteins[d]	Not inhibited	Inhibited
Amino acid sequence identity[e]	B. thermoproteolyticus	S. marcescens
Amino acid composition resembles[f]	B. thermoproteolyticus thermolysin	S. marcescens metalloprotease

[a] See Fig. 2.
[b] Estimated by isoelectric focusing (See Chapter II).
[c] See Table 3.
[d] See Table 2.
[e] See Fig. 3.
[f] See Table 4.

1990), although Tseng and Mount (1973) described a role for a protease in the lysis of plant protoplasts. However, our results showing a 20 to 30% loss of maceration ability due to mutation provides direct evidence that proteases contribute to soft rot pathogenesis. Dahler et al. (1990) did not find significant differences in maceration on potato tuber disks or chrysanthemum (Dendranthema grandiflora Tzvelev.) stems and whole plants using protease-deficient mutants of E. chrysanthemi. Other researchers have not obtained significant reductions in maceration with enzyme-deficient mutants of erwinias (Hinton et al., 1989; Payne et al., 1987). Besides technical differences in maceration assays, it may also be possible that multiple or specific proteases are required for maceration.

Although the function of proteases in maceration is not clear, J. Lewosc, A. Kelman, and L. Sequeira (University of Wisconsin, Madison and North Carolina State University, personal communications) have suggested that Ecc strain SR394 protease (pI 4.8, 43 kDa; similar to Prt2) is involved in the degradation of plant cell wall extensins. The expression of prt1 in planta (at 12 to 15 h) indicates that Prt1 would be available for the digestion of soluble extensins at the time (8 to 24 h) extensins are induced in wounded potato tubers (Rumeau et al., 1990); accumulation begins at 8 h from wounding and peaks after 24 h. Some further accumulation of extensin mRNA was reported in Ecc-challenged, wounded potato tuber tissues compared to wounded, non-inoculated tissues (Rumeau et al., 1990). However, no consistent pattern emerged or novel transcripts of extensin were induced by Ecc.

Potato tubers also contain high levels of wound-inducible protease inhibitors, which may be targeted against proteases secreted by invading pathogens. However, the Ecc extracellular protease(s) is not inhibited by these protease inhibitors (Heilbronn and Lyon, 1990). Our results (not shown) support this conclusion; potato tuber extracts did not inhibit gelatin-degrading Ecc proteases. Thus, Ecc extracellular protease(s) may represent components of the Ecc arsenal for pathogenesis to which the host does not respond.

Because of possible nutritional advantage to Ecc provided by proteases, we assessed their role during _in planta_ growth. Slower _in planta_ growth of Ecc mutant L-957, a _prt_1 site replacement mutant, and L-763, a Tn_5_ mutant, suggests that reduced potato tuber maceration may be due to the decreased growth, and, consequently, to decreased production of extracellular enzymes. Slower growth was only evident after 24 h suggesting a more important role for proteases as nutrients become limiting. Thus, extracellular proteases may help Ecc to use maceration products to supply nitrogen and carbon for bacterial growth. Unexpectedly, mutant L-763, containing a Tn_5_ transposon in an unknown location affecting the production of both Prt1 and Prt2, did not cause any less maceration than L-957. Thus, although this mutant did not express any protease in gelatin plate assays, it may have some expression of one or both enzymes _in planta_ (Kelemu and Collmer, 1990). Alternatively, Prt2 may have no important role either in maceration or growth rate. Since the mutation in L-763 affects virulence by a different manner from L-957, studies of this mutant will be helpful in

further understanding the roles for multiple proteases in soft rot pathogenesis.

Our studies on expression of the different proteases in planta were hampered by difficulties in recovering protease activity from the plant tissue. This was overcome by measuring mRNA production in planta, which showed that prt1 mRNA was induced by the presence of plant tissue. Biphasic prt1 mRNA accumulation may be explained in two ways; it reflects the bacterial growth in the first minor peak (2 to 4 h) and induction by plant cell wall degradation products in the major peak (12 h). Alternatively, it may indicate that the induction of two protease genes, an early gene (2 to 4 h) and a second gene (12h) that both hybridize to the prt1 probe. The second hypothesis is supported by the internal controls of bacterial gene probes for pectic enzymes that do not show biphasic, growth-related accumulation profiles. However, the Southern analysis with prt1 probe hybridized to only one DNA fragment arguing against a second, prt1-like protease (Chapter II). Otherwise, the transient induction pattern of prt1 was similar to that of previously obtained for other Ecc cell wall degrading enzymes (Yang, 1990) and resembled especially the kinetics of endo-polygalacturonase expression. This similarity of prt1 transcription to those of other cell wall degrading enzymes in tuber maceration suggests that Prt1 is involved in pathogenesis. Thus, among the other in planta induced genes, the analysis of the prt1 promoter may be useful for studying regulatory elements involved in the pathogenicity.

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IV. CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

The objective for this dissertation was to characterize an Erwinia carotovora subsp. carotovora (Jones) Bergey et al. (Ecc) extracellular protease and elucidate its role in potato (Solanum tuberosum L.) soft rot. For this purpose a gene, prt1, coding for a protease was subcloned and sequenced. Its deduced amino acid sequence shows similarity (>50% identity) to many bacterial metalloproteases, including proteases from several human pathogens and some soil bacteria. Sequence identity was greatest with Bacillus thermoproteolyticus Rokko thermolysin. Prt1 was not expressed in Ecc under in vitro conditions, and may be expressed chiefly during growth in planta; mRNA accumulation for Prt1 peaked approximately 12 to 15 h after inoculation with Ecc on potato tuber slices. Escherichia coli (Migula) Castellani and Chalmers-produced Prt1 had a molecular mass of 38 kDa, pI of 4.8, and was a metalloprotease. To assess the significance of Prt1 in pathogenesis, a protease-deficient mutant of Ecc was constructed by marker exchange mutagenesis and used for tuber maceration assays. This mutant, L-957, macerated potato tuber slices 20 to 30% less and grew slower in planta compared with the wildtype pathogen.

The possibility that another extracellular protease occurs in Ecc was raised when the major extracellular protease was purified from culture supernatants of Ecc grown in PEM and characterized. The NH_2-terminal sequence of the purified protease did not match the deduced sequence of prt1, but showed significant identity to the NH_2-termini of

Erwinia chrysanthemi Burkholder et al. and Serratia marcescens Bizio extracellular proteases. This protease, Prt2, had a molecular mass of 43 kDa, a pI of 4.8, a pH optimum at 7.5, a temperature optimum at 40°C, and did not reduce potato tuber maceration as compared to wildtype Ecc.

FUTURE DIRECTIONS

The existence of two proteases is not surprising considering the presence of multiple proteases in several animal and plant pathogenic bacteria. However, it would be interesting to know the roles for the individual proteases in pathogenesis. Since both proteases are active on gelatin, they may have similar substrate specificities. Their regulation, however, seems to differ; Prt1 is mainly expressed in planta, while Prt2 can be produced in the absence of plant material. For future work, cloning and sequencing of the gene encoding Prt2 will allow comparison of the two proteases. The differential regulation of the two proteases suggests differences in the upstream sequences that may contribute to the different expression patterns. Further regulation studies can be performed with the protease-deficient mutant, L-763. Since this mutant does not produce either protease, Tn5 may have inactivated a positive regulator for both of the proteases. This would indicate overlapping regulation between the two proteases.

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VI. APPENDICES

APPENDIX A

CONSTRUCTION OF DELETION CLONES FOR SEQUENCING

1. Restrict. Digest 200 to 300 ng DNA/timepoint with the first restriction enzyme to produce a 3'-overhang. This end will not be digested by exonuclease III. Check digestion on an agarose gel.
2. Restrict. Digest with a second restriction enzyme to produce a 5'-overhang or blunt end. These 3'-ends are susceptible to exonuclease III digestion.
3. Remove protein (optional). Extract with phenol:chloroform (1:1, v/v).
4. Ethanol precipitate. Add 0.5 volumes of ammonium acetate and 2.5 volumes of ethanol, precipitate at -20°C for 30 min to overnight. Centrifuge at 13,000 x g for 15 to 45 min at 4°C, discard supernatant, and wash precipitate with 70% ethanol. Resuspend in sterile water to dissolve the pelleted DNA.
5. Exonuclease III reaction buffer volume. Combine DNA and 2X exonuclease III buffer (previously prepared and stored at -20°C). Equilibrate at 37°C for 15 min. The total reaction volume = 3 µl X number of time points. Exonuclease III digests roughly 200 bp/min at 37°C from the 3' blunt or recessed strand.
6. Start the exonuclease III digestion. Add exonuclease III; use approximately 20 U of exonuclease III/µg DNA. Remove 3 µl aliquots every 30 sec to tubes containing 2 µl 10X exonuclease VII buffer, mix, and place into 70°C heating block for 10 to 15 min to stop the

reaction. Exonuclease VII buffer contains EDTA, which inhibits exonuclease III activity.

7. Exonuclease VII digestion. Add 14 µl sterile water, 1 µl exonuclease VII (2U), and incubate at 37°C for 45 min. This step removes any ssDNA.
8. Ethanol precipitation. As described in step 4 above.
9. Blunt ending. Resuspend each pellet in 14 µl sterile water, add 1 µl 4.5 mM dNTPs, 2 µl nick translation buffer, 2 µl 10 mM dithiothreitol, and 1 µl Klenow fragment of DNA polymerase (2U). Mix and incubate at room temperature for 30 min. This step will fill in any remaining staggered ends, since exonuclease VII does not remove single stranded DNA "cleanly."
10. Check deletions on agarose gel. Run 10 to 15 µl on 0.7% agarose gel to screen for desired sizes using appropriate molecular mass markers.
11. Ligation and transformation. Ligate the rest of the sample and transform into E. coli (Maniatis et al., 1982). Deleted DNA for ligations can also be obtained from low-melting point agarose gel (0.7%; SeaPlaque). After electrophoresis, cut appropriate bands out of the gel and ligate according to Struhl (1986).

2X exonuclease III buffer	10X exonuclease VII buffer
100 mM Tris-HCl, pH 8.0	670 mM KPO_4
10 mM $MgCl_2$	83 mM Na_2EDTA
40 mM ß-mercapthoethanol*	200 mM ß-mercapthoethanol*

*Add ß-mercapthoethanol just before use.

APPENDIX A

10X nick translation buffer

0.5 M Tris-HCl, pH 7.2
0.1 M $MgSO_4$
500 ug/ml BSA
10 mM dithiothreitol

APPENDIX B

CHARACTERIZATION OF Ecc STRAIN L-763,

A TRANSPOSON MUTANT DEFICIENT IN PROTEASE PRODUCTION

Southern hybridization. A protease-deficient mutant of E. carotovora subsp. carotovora (Jones) Bergey et al. (Ecc) strain EC14 was constructed by Tn5 mutagenesis (Allen et al., 1986) and designated strain L-763. To determine if the 5.7 kb Tn5 was inserted within prt1, Southern blots were performed. The probe for prt1 was a 2.6 kb HincII fragment of pSK1 (Fig. 1 of Chapter II). This fragment contains 797 bp from the 3' end of the prt1 plus approximately a 1.8 kb region downstream of prt1. The fragment was purified from 0.7% low melting agarose (SeaPlaque; FMC) using a GeneClean kit (Bio 101, Inc., La Jolla, Ca.). Plasmid pBR322::Tn5 was used as a probe for Tn5.

Chromosomal DNAs from Escherichia coli (Migula) Castellani and Chalmers strain HB101, wildtype Ecc (EC14), and L-763 were isolated by the Marmur (1960) procedure as modified for the PPWS 5014 laboratory protocol. Approximately 5 µg DNA was digested overnight with EcoRI or HindIII, precipitated with 0.1 volumes of 3 M sodium acetate and 0.6 volumes of isopropanol, and incubated for 30 min to overnight at room temperature. DNA was centrifuged at 13,000 x g for 15 min at room temperature to pellet the DNA. The dried DNA pellet was dissolved in 10 to 15 µl of TE. Plasmids pSK1 and pBR322::Tn5 were linearized with EcoRI and used as positive controls for prt1 and Tn5, respectively.

Agarose gel electrophoresis and transfer to membrane were done

according to Maniatis et al. (1982). The probes and 1 kb DNA ladder (Bethesda Research Laboratories; BRL) used as molecular mass markers were labelled with biotin (BluGENE Nonradio-active Nucleic Acid Detection System; BRL). Hybridization and detection were by BluGENE (BRL) labelling and detection kits.

In EcoRI digests, the prt1 probe hybridized to 5.5 and 6.5 kb fragments in both Ecc and L-763 (Fig. 1). No shift in the migration of L-763 EcoRI fragments compared to Ecc indicates that Tn5 was not inserted in prt1. The appearance of two fragments is expected since an EcoRI site occurs in prt1 (Fig. 1 in Chapter II). This endonuclease cleaves the 2.6 kb HincII fragment into 0.55 and 2.5 kb fragments. Light bands probed with prt1 were occasionally detected at 0.5 and 1.5 kb in EcoRI digests. This may be due to hybridization to the 3' end of the probe that does not contain the prt1. The Tn5 probe hybridized with a >15 kb fragment in L-763 and thus differs from the bands detected with the prt1 probe. When DNA was digested with HindIII, a 15 kb fragment was observed. These results suggest that the Tn5 insertion in L-763 is in a region regulating prt1, in a second protease gene, or in a gene required for protease secretion.

SDS-PAGE. Extracellular protein production of L-763 was compared to Ecc to determine if the mutant lacks any extracellular proteins. Ecc and L-763 were grown in PEM at 30°C with orbital shaking (200 rpm) overnight. The proteins in the culture supernatant were precipitated with 80% ammonium sulfate. Approximately 20 μg of proteins were run on a 12.5% SDS-PAGE gel according to Laemmli (1970). To localize proteins

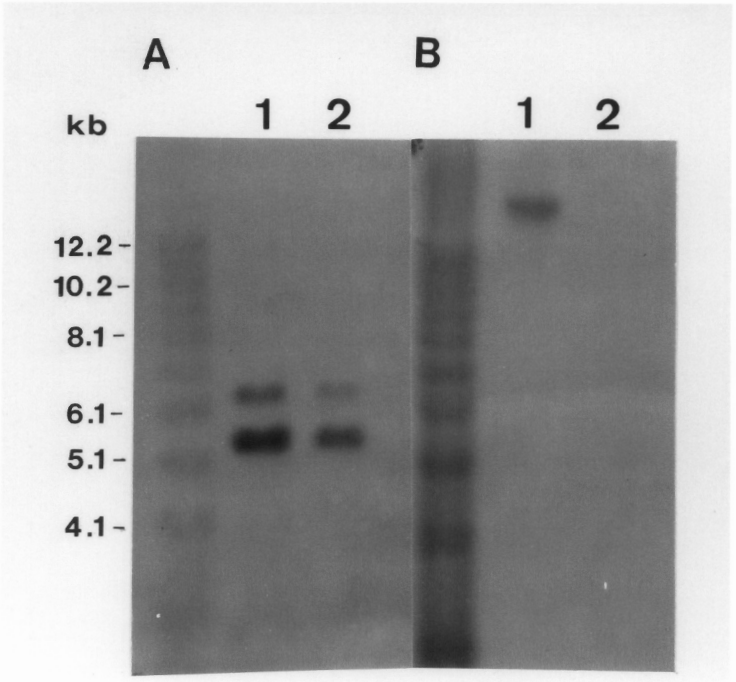


Fig. 1. Southern analyses of E. carotovora subsp. carotovora (Ecc; lane 1) and Ecc transposon mutant L-763 (lane 2) genomic DNA hybridized with (A) biotin-labelled prt1 probe and (B) pBR322::Tn5.

expressing protease activity on gelatin, the SDS-PAGE gel was incubated with a gelatin overlay after electrophoresis (Chapter II). The proteins were stained with Coomassie Blue (Schleif and Wensink, 1981).

The protein profile from L-763 differed from Ecc. The protein band (43 kDa) corresponding to protease identified by gelatin overlay was less densely stained with Coomassie blue in the mutant, but not completely absent, compared to the wildtype. This may be due to reduced protease production or the presence of another protein with similar molecular mass. No protease activity was observed in the mutant by gelatin overlay. Further, two protein bands with molecular masses of 32 and 36 kDa detectable in the wildtype were absent in L-763.

Further studies are required to determine if Tn*5* in L-763 is located in a transport protein, a gene encoding a positive regulatory protein, or a protease not detectable with gelatin as a substrate. If protein transport is affected by Tn*5*, protease would be expected to accumulate in the cytoplasm. Determination of protease accumulation by protease activity assay is not possible because of a putative cytoplasmic protease inhibitor. However, pectate lyase secretion by L-763 appears unaffected as detected by PEC-YA plates. Further, the mutant transformed with a plasmid (pSK23) containing *prt*1 secretes protease as determined by gelatin detection plates suggesting that the Tn*5* is not affecting protein transport. Tn*5* insertion into a positive protease regulatory element could explain the lack of protease activity in the mutant. Since the mutation affects production of other proteins, based on missing proteins in the mutant, it could represent a global

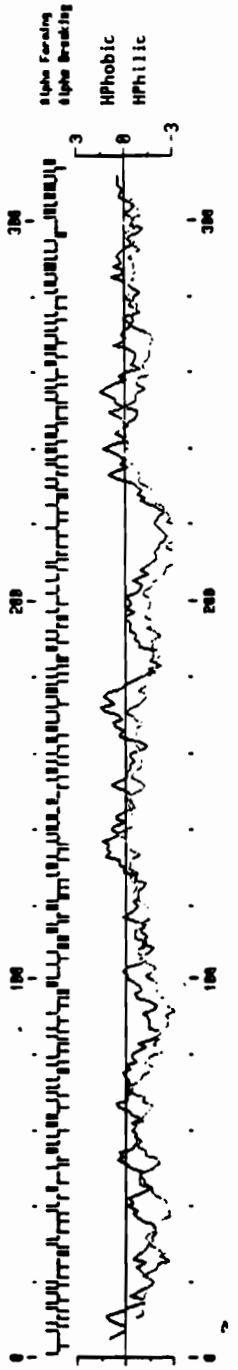
regulator. The third possibility, a Tn*5* inserted into a second protease, does not explain the different protein profile of the mutant compared to wildtype.

APPENDIX C

HYDROPHOBICITY PLOT OF THE DEDUCED POLYPEPTIDE FROM prt1

Fig. 1. Hydrophobicity plot of the deduced polypeptide from prt1. The hydrophobicity plot, created by the University of Wisconsin Genetics Computer Group sequence analysis software, is calculated by the Kyte and Doolittle (1982) algorithm. Numbers indicate amino acid residues in the polypeptide.

APPENDIX C


Alpha Forming
Alpha Breaking
HPhobic
HPhilic
3
0
-3
0
100
200
300

APPENDIX D

EFFECTS OF pH AND IONS ON PRT2 ACTIVITY

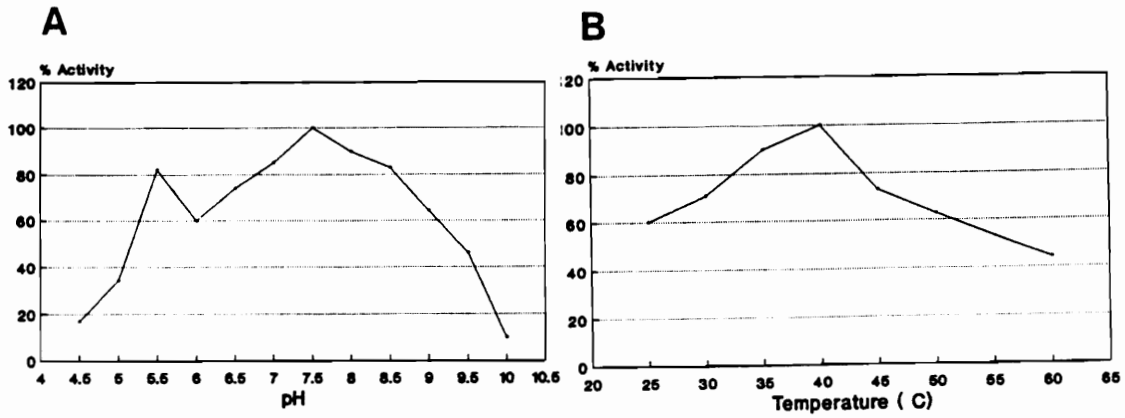


Fig. 1. pH (A) and temperature (B) optima of E. carotovora subsp. carotovora Prt2. Protease assays using azocasein as substrate were performed as described in Chapter III.

Table 1. Effect of cations on E. carotovora subsp. carotovora (Ecc) Prt2 activity

Addition	Concentration (mM)	Activity (%)[a]
None	-	100
EDTA	1	68
$CaCl_2$	10	104
$ZnCl_2$	10	31
$MgCl_2$	10	80
$CoCl_2$	10	28
$CuCl_2$	10	3

[a] Concentrated culture supernatant of PEM-grown Ecc was preincubated with 1 mM EDTA to chelate endogenous cations at 30°C for 20 min. Various cations were added to the sample and incubated further under the same conditions for 20 min to test their ability to restore protease activity. Protease activity measured using azocasein as substrate is expressed as percentage of control activity with no EDTA added.

APPENDIX E

LECTIN BINDING ASSAY FOR PRT2

Glycosylation was examined as a possible explanation for the different electrophoretic migration of Prt1 (38 kDa) and Prt2 (45 kDa) on 12% SDS-PAGE (See Fig. 1 in Chapter III). Anomalous electrophoretic migration has been observed for a Bacillus subtilis (Ehrenberg) Cohn metalloprotease (Yang et al., 1984) and for erwinia pectate lyases (Ito et al., 1988; Keen and Tamaki, 1986; Lei et al., 1987; 1988; Tamaki et al., 1988). The hypothesis that high pI proteins migrate more slowly (Keen and Tamaki, 1986) was not supported by a slow migrating acidic pI protein (Tamaki et al., 1986). Thus, protein glycosylation may explain anomalous electrophoretic migration (Segrest et al., 1971; Sharon and Lis, 1975). Although glycosylated prokaryotic proteins have been reported rarely (Kamat et al., 1987; Pfannenstiel et al., 1987; Srivastava, 1984), Erwinia carotovora pectate lyases are glycosylated (A. Kelman, North Carolina State University; personal communication; Suguira et al., 1984) raising the possibility that Ecc proteases may also be glycosylated. Glycosylation may account for the lack of denaturation of Prt2 in samples boiled with SDS and ß-mercaptoethanol, since activity is detectable by gelatin overlay. Under similar conditions, no activity is observed for Prt1 produced by E. coli, which does not glycosylate proteins.

The glycosylation of Prt1 has not been tested. However, three putative glycosylation sites (Asn-X-Thr/Ser) were observed within the

deduced amino acid sequence of prt1 at residues 21 to 23, 60 to 62, and185-187.

Prt2 was tested for glycosylation using biotinylated lectins. The lectins tested were wheat (Triticum aestivum L.) germ agglutinin (WGA; BioCarb Chemicals, Lund, Sweden), peanut lectin (Arachis hypogaea L., PNA; Sigma), and castorbean lectin (Ricinus communis L., RCA; Sigma). Purified extracellular protease (10 µg) was separated by 12% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell). The membrane was blocked with 10 mM Tris-HCl (pH 7.5), 0.9% NaCl, and 4% BSA with shaking (10 rpm) at room temperature for 1 h. Incubation with lectins (30 µl/ml) was performed in 6.7 mM KH_2PO_4, 0.15 NaCl, and 3% BSA with shaking (10 rpm) at room temperature overnight. After incubation with lectins, the membranes were washed several times with distilled water and incubated with 2% BSA with shaking (10 rpm) at room temperature for 15 min. Bound lectin was localized with avidin peroxidase conjugate (30 µl/ml) with shaking (10 rpm) at room temperature for 2 hr. The bands detected with the lectins tested were very faint (wheat germ, peanut, and castorbean); among these the peanut lectin gave the strongest signal (Fig. 1).

APPENDIX E

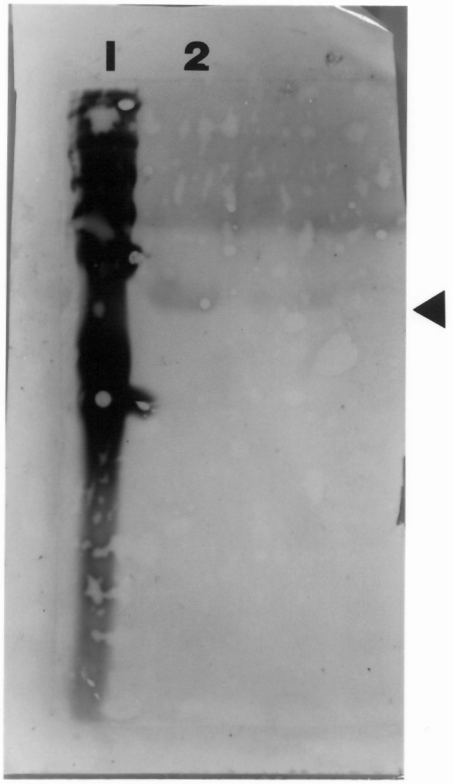


Fig. 1. Peanut lectin binding to Prt2 from E. carotovora subsp. carotovora. Molecular mass standards (lane 1) and 10 μg Prt2 (lane 2) are indicated. The arrow shows the location of Prt2. Method as described in the text.

APPENDIX F

INHIBITION OF Ecc PROTEASE BY POTATO EXTRACTS CONTAINING PROTEASE INHIBITORS

Potato tubers are known to contain several protease inhibitors that may be used as a defense mechanism against extracellular proteases secreted by invading pathogens (Ryan, 1973). A test was performed to determine if tuber tissue contains protease inhibitors capable of inhibiting Ecc extracellular protease (Prt2). Approximately 3 to 5 g of potato tuber tissue was ground in 1.0 ml of 0.1 M Tris-HCl (pH 8.0) with mortar and pestle at 4°C. The ground tissue was filtered through three layers of cheese cloth and 10 µl of the filtrate, containing soluble protease inhibitors, was added to different concentrations of Ecc extracellular proteins (0.0012 to 1.2 µg) containing Prt2 in a total volume of 20 ul. After the extract was incubated with Ecc extracellular proteins at 30°C for 20 min, 10 µl of the mixture was applied on gelatin overlay (Chapter II) and the overlay was incubated at 30°C for 1 to 2 h; any protease activity was detected by measuring clearing zones in $HgCl_2$-precipitated gelatin (Chapter II). None of the concentrations of potato soluble proteins inhibited Prt2 activity compared to the control with no added potato proteins. This is consistent with the results of Heilbronn and Lyon (1990); purified potato chymotrypsin inhibitor did not inhibit Ecc strain 177 extracellular protease activity.

APPENDIX G.

SOUTHERN ANALYSIS OF prt2

The objective was to confirm that the purified E. carotovora subsp. carotovora extracellular protease (Prt2) was encoded by a different gene from the protease gene subcloned from cosmid pCA7 (prt1).

Construction of prt2 probe. A nucleotide probe for prt2 was constructed that corresponds to the NH_2-terminal sequence of purified Prt2 (AXGTGYSDVYDLYNYHCRGD). The first primer was backtranslated from the amino acid sequence (residues 4 to 11) TGYSDVYD. The codons for each amino acid were selected according to the most preferred codons in the deduced prt1 amino acid sequence and E. carotovora pectate lyase gene (Lei et al., 1987) and resulted in the following sequence:

```
    T   G   Y   S   D   V   Y   D
5' ACC GGC TAT TCC GAT GTG TAT GAT 3'   24 b
```

For preparing the primer a complementary sequence was used:

5' ATC ATA CAC ATC GGA ATA GCC GGT 3' (PRT2 primer 1)

A degenerate oligonucleotide primer was also constructed, because the first primer did not hybridize selectively; this was probably due to incorrect codon usage. The second primer was a 27-base oligonucleotide corresponding to the conserved amino acid sequence DVYDLYNYH (residues 8 to 16) and used all the possible codons for the selected amino acids:

```
    D   V   Y   D   L   Y   N   Y   H
5' GAT GTT TAT GAT CTA TAT AAC TAT CAT 3'  (PRT2D primer 2)
     C   C   C   C T T   C   T   C   C
         G           G
         A           C
```

Again, the complementary sequence was synthesized because of its potential usefulness for both Northern and Southern hybridizations:

```
5' ATG GTA GTT ATA TAA ATC GTA TAC ATC 3'
   G   A   A   G   G G G   A   A   G
                   C           C
                   A           G
```

Southern analysis Chromosomal DNA was isolated according to Marmur (1960); digested with EcoRI, SmaI, and EcoRI plus SmaI; and the resulting fragments were separated electrophoretically on a 0.7% agarose gel. DNA was transferred to a nylon membrane (Nytran; Schleicher and Schuell, Inc., Keene, NH) by blotting, and prehybridized and hybridized at 37° overnight according to Maniatis et al. (1982). The membranes were washed with increasing stringency using solutions containing tetramethyl-ammonium chloride to increase A to T pair binding strength as described by Wood et al. (1985). A 22-base oligonucleotide (5'-TCGTATTATCGCAAACGGCACC-3') constructed from prt1 (Chapter II) was used as a probe for that gene. The 24-base and 27-base oligonucleotides based on the NH_2-terminus of the Prt2 amino acid sequence were synthesized by Dr. M. Lederman (Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, Va.) and used as a probe for prt2. The oligonucleotides were labelled with ^{32}P using T4 polynucleotide kinase (Maniatis et al., 1982). Unincorporated label was removed by gel filtration following the manufacturer's instructions (G-25 column; 5 Prime → 3 Prime, Inc., West Chester, Pa.).

Results The prt1 probe hybridized as expected to the 5.5 kb EcoRI

fragment, 8.0 kb SmaI fragment, and 1.0 kb SmaI-EcoRI fragment. No faint bands to other putative, similar proteases were detected. Hybridizations with the prt2 (primer 1 or 2) probe did not show any bands. It is not clear why the degenerate primer showed no hybridization.

VII. VITAE

Sirkka R.M. Kyöstiö was born on December 5, 1962 in the busy, bustling city of London, England. After three years of experiencing the London fog, her mother took her to the refreshing air of Oulu, a town located close to the Arctic Circle in Northern Finland. Except for six months in exotic Kabul, Afghanistan, her family lived the next ten years in Oulu and she got used to long, light summer nights and short, snowy winter days. The family then moved to Hausjärvi, a small agricultural village in southern Finland. She graduated from Hausjärvi High School in 1981, and was then accepted into the Biology Department at the University of Helsinki. After three years of general biology, she specialized in Plant Physiology. Her thesis research, "Carboxylating enzyme activities in filamentous fungi, Schizophyllum commune," was supervised by Dr. Marjatta Raudaskoski. As a part of her Masters studies under the International Student Exchange Program, she attended Virginia Polytechnic Institute and State University (VPI&SU), USA, in 1985-86, and had an opportunity to work with Dr. Richard E. Veilleux on plant cell and tissue culture. She received her Master's (Filosofian Kandidaatti) in spring 1987. Determined to learn molecular biology techniques, she flew across the Atlantic back to VPI&SU, and started her Ph.D. studies on potato soft rot under supervision by Drs. George H. Lacy and Carole L. Cramer.