RELEVANCE OF THE GALACTURONIC ACID UPTAKE SYSTEM TO THE VIRULENCE PHENOTYPE OF *Erwinia chrysanthemi* EC16

by

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ABSTRACT

Erwinia chrysanthemi EC16 is a plant pathogen responsible for soft-rot disease in many plant species. Pathogenicity appears to be chiefly due to pectolytic activity. Pectin-degrading enzymes break down the plant cell wall into monomers, saturated and unsaturated dimers and oligomers of galacturonic acid (GA) as well as keto-deoxy-gluconates. The monomers and dimers are taken up by the bacterium to be utilized for carbon sources, energy, and pectinase induction. The uptake of these compounds must then play a crucial role in the virulence of the bacterium. A 3.4-kb *EcoRV* fragment of EC16 DNA capable of complementing an *exuT* mutant was cloned into pUC19. Alkaline phosphatase gene fusions and complementation analysis suggest that the entire 3.4-kb are required for complementation of a GA uptake mutant.

EC16 GA transport mutants were generated using marker exchange-eviction mutagenesis and assessed for their significance in pathogenicity. Utilizing an *nptI-sacB-sacR* cartridge the *exuT* gene was inactivated and exchanged with the *E. chrysanthemi* genome. The cartridge was then evicted. The resulting mutants were *exuT*$^-$ containing a 1.7-kb deletion in the *exuT* gene. The mutants demonstrated lower transport activity for GA resulting in a reduced growth rate on GA as the sole carbon source. This phenotype did not reveal a significant difference in the virulence phenotype.

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CHAPTER I

INTRODUCTION TO DEGRADATION AND CATABOLISM OF PECTINACEOUS COMPONENTS OF THE PLANT CELL WALL IN ERWINIA CHRYSANTHEMI

Erwinia chrysanthemi is a bacterial plant pathogen responsible for soft-rot disease in a variety of plant species. A prerequisite for a successful pathogen is its ability to invade its host. Soft-rot *Erwinias* secrete several extracellular enzymes that can attack components of the plant cell wall: pectinases, cellulases, hemicellulases, and proteases. The search for the molecular factor(s) that enables *E. chrysanthemi* to breach the plant cell wall and cause soft-rot disease has led repeatedly to the pectic enzymes they secrete (Basham and Bateman, 1975). Since then, the key focus of research has been the genetic and regulatory factors affecting the production of these enzymes. Degradation of the pectinaceous component of the plant cell wall releases monomers, dimers, and oligomers of galacturonic acid (GA) and keto-deoxy-gluconates (Garbaldi and Bateman, 1971; Preston *et al.*, 1992). These compounds are then taken up by the bacterium and utilized through catabolism for carbon sources, energy, and pectinase induction (Collmer and Bateman, 1982; Hugouvieux-Cotte-Pattat *et al*, 1983; Reverchon *et al.*, 1992). Although pectate lyases have been shown to be induced by catabolites of GA or dGA, these molecules must first enter the bacterial cell for intracellular catabolism to occur. Therefore, transport of degraded plant cell wall components is necessary and must be an important part in the virulence phenotype of *E. chrysanthemi*. *E. chrysanthemi* is an ideal model for studying pathogenesis because of its ability to cause disease in a variety of plants and ease of genetical manipulation. Mutant studies in *E. chrysanthemi* may serve to understand the mechanisms of virulence of phytopathogenic fungi that express similar plant degradative activities.

Cell wall degradation and catabolism

E. chrysanthemi, a facultatively anaerobic Gram-negative bacillus, is a plant pathogen responsible for soft-rot disease of many plant species. Soft-rot disease is characterized by maceration and killing of susceptible parenchymatous tissue of plants (Basham and Bateman, 1975). Pathogenicity appears to be chiefly due to pectolytic activity. *E. chrysanthemi* secretes five major isozymes of pectate lyases (Condemine *et al.*, 1986) that cleave polygalacturonic acid (PGA) (Table 1). PGA is a homopolysaccharide made up of unbranched chains of α-(1,4)-linked D-galacturonic acid residues. Pure galacturonans occur infrequently, and several neutral sugars and other constituents are now considered to be integral components of pectins (Starr and Chatterjee, 1972). Pectin (methylated polygalacturonic acid) (Figure 1) is a major component of the plant cell wall with a glue-like function that helps hold cells together. The plant cell wall is made largely of cellulose arranged in layers around the cell's plasma membrane. Plant cell walls normally have three parts: the middle lamella, which is the first part to form when the cells divide and is composed primarily of pectin; the primary cell wall; and the secondary cell wall, which is composed of cellulose and lignin (Figure 2). Degradation of components of the plant cell wall results in loss of turgor pressure and eventual lysis of the cell. This gives infected tissue the characteristic watery look of soft-rot disease.

Pectin is cleaved by two different types of enzymes: hydrolases and lyases. *E. chrysanthemi* produces one known hydrolase, but the primary degradation of polygalacturonic acid (PGA) is by the lyases producing 4,5 unsaturated digalacturonic acid (udGA). The resulting digalacturonic acid is transported into the bacterial cell where it is further degraded to pyruvate and triose phosphate (Figure 3) (Reverchon *et al.*, 1991). The genes corresponding to the five pectate lyases (*pelA-E* genes) and to the pectin methylesterase (pem) have been identified and are organized in two clusters on the 3937 (French strain) chromosome (Hugouvieux-Cotte-Pattal *et al.*, 1989). Many genes

Table 1. Extracellular enzymes produced by *Erwinia chrysanthemi*.

Enzyme Name	Gene	Substrate	Products
endo-Pectate lyase	*pelA*	PGA	predominant oligomers: di-to dodecamers
endo-Pectate lyase	*pelB*	PGA	predominant oligomers: tri-tetramers
endo-Pectate lyase	*pelC*	PGA	predominant oligomers: tri-tetramers
endo-Pectate lyase	*pelD*	PGA	not determined
endo-Pectate lyase	*pelE*	PGA	predominant oligomers: dimers
exo-Polygalacturonase	*ExoPeh*	PGA	dimers
Pectin methylesterase	*Pem*	Pectin	Pectate

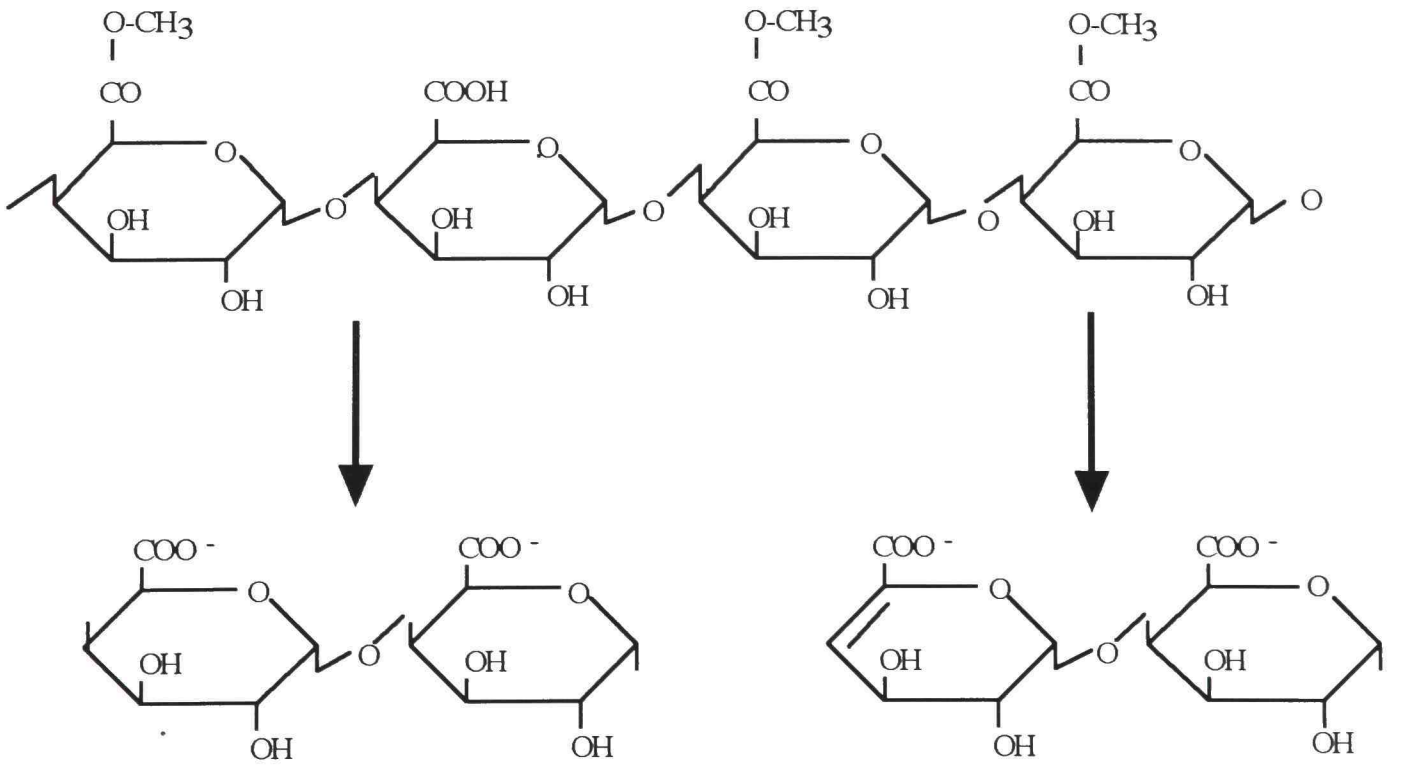

Figure 1. Schematic of pectin molecule. Pectin is a major component of the plant cell wall material and its degradation leads to maceration of plant tissue. Pectin methylesterase and pectate lyases cleave pectin to generate digalacturonic acid and unsaturated digalacturonic acid.

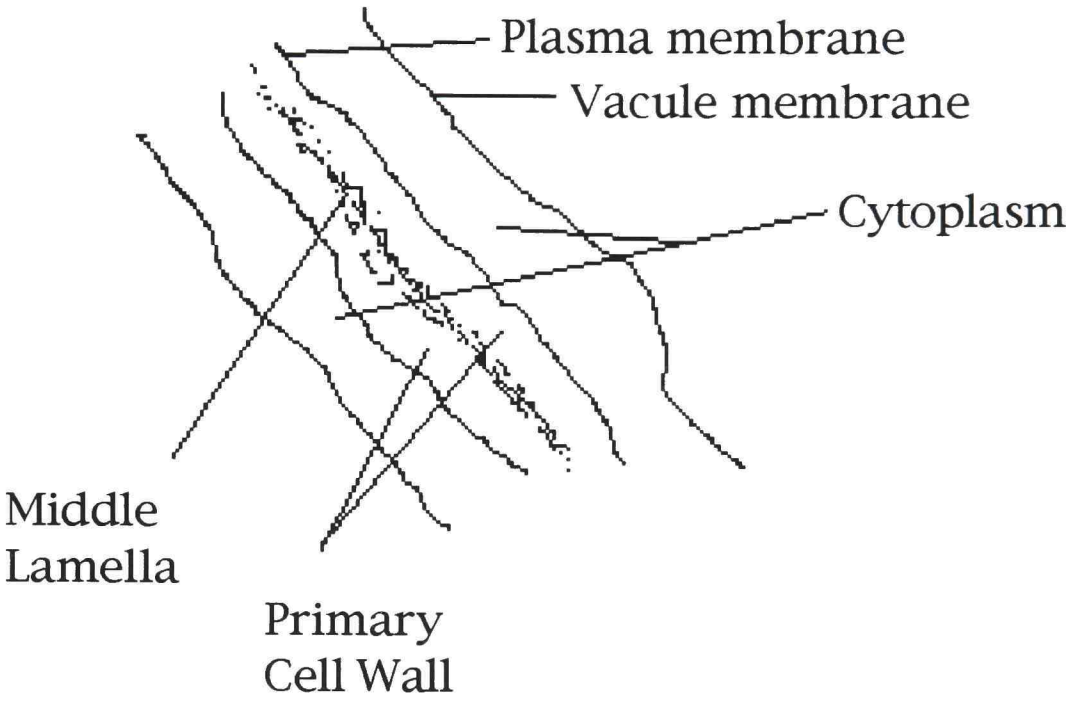

Figure 2. Schematic of plant cell. Galacturonans are structural polymers in plant cell walls. According to current models, the cell wall structure is comprised of cellulose fibers ensheathed in hemicellose polymers that are interconnected by pectic polymers. The pectic polysaccharides, which also prevail in the middle lamella, are characterized by the predominance of galacturonosyl residues (Collmer et al., 1982).

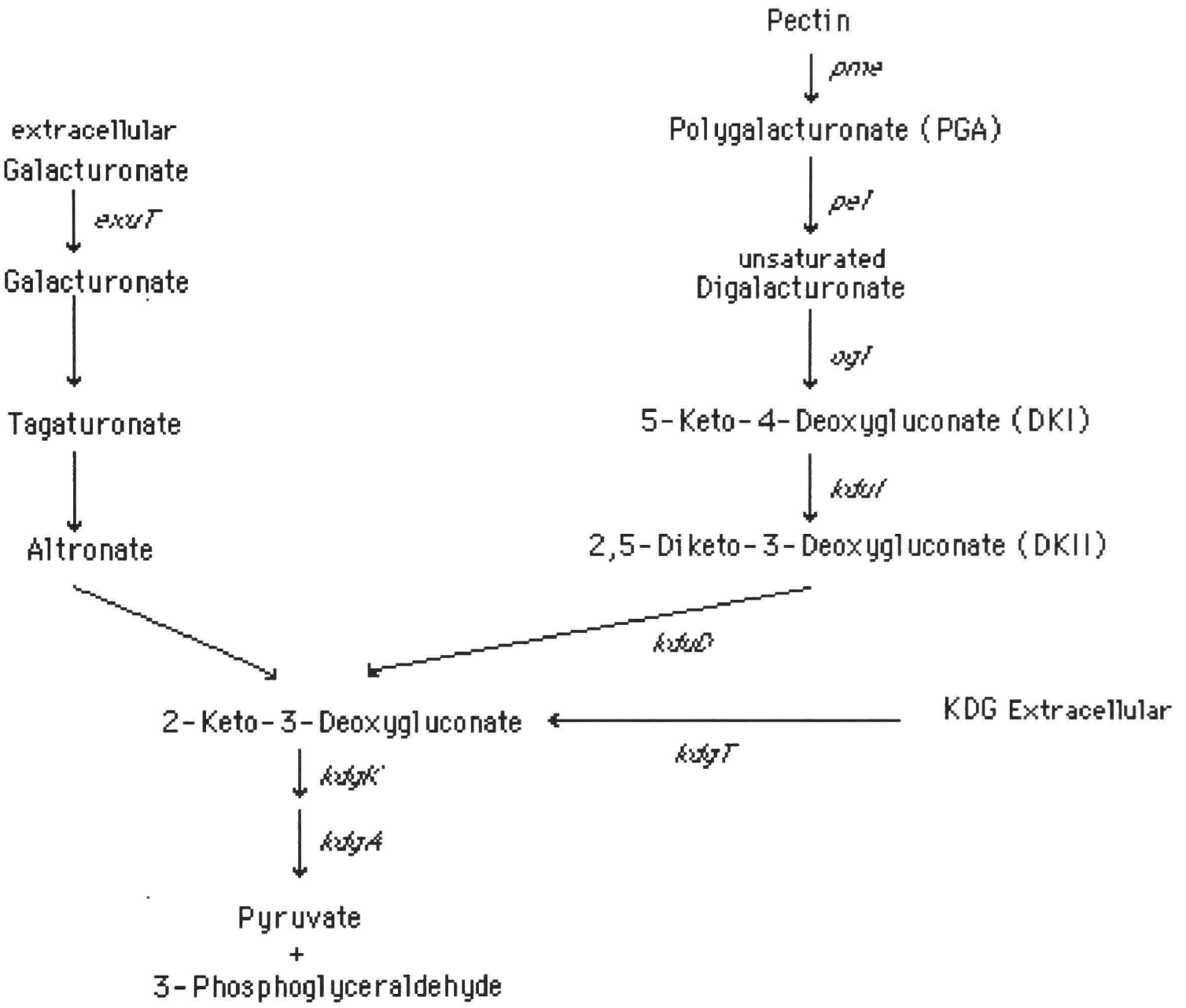

Figure 3. Degradative pathway of galacturonic acid and pectin in E. chrysanthemi. The different steps are catalysed by the products of the specified genes. The transport system mediating entry of galacturonic acid into the cell is indicated exuT. Break down of pectin and PGA by pectate lyases leads essentially to unsaturated digalacturonic acid.

responsible for the degradation and utilization of pectin and PGA are known to be induced with the presence of PGA or GA. These include the *pelA-E*, *ogl* (oligogalacturanate lyase) and *kdgT* (KDG uptake). The intracellular inducers, 2-keto-3-deoxygluconate (KDG), 2,5-diketo-3-deoxygluconate (DKII), and 5-keto-4-deoxyuronate (DKI) are products of metabolism (Chatterjee *et al.*,1985; Condemine *et al.*, 1986). Chatterjee *et al.* (1979) reported a several-fold higher accumulation of extracellular pectate lyases during incubation with galacturonan than during incubation with glycerol, gluconate, or galacturonic acid. Investigations of the ability of *E. chrysanthemi* to transport undegraded galacturonan is complicated, and evidence suggests that the polymer cannot be taken up (Collmer and Bateman, 1982). However, due to the increased production of pectate lyases, the bacterium must be able to detect the presence of the polymer in its environment.

E. chrysanthemi secretes basal levels of pectate lyases to rapidly release dimers, trimers and oligomers from galacturonans in its environment and responds to these dimers with increased production of pectate lyases and exopolygalacturonase synthesis (Collmer *et al.*, 1982). The bacterial cells are then somehow able to distinguish between the monomers and a polymer that requires extracellular digestion. It has been noted that the extracellular pectic enzymes do not release monomers of galacturonic acid as a major product and that the monomer is inferior to either of the dimers as an inducer of pectate lyases (Collmer and Bateman, 1981). The key to understanding soft-rot disease is to understand and identify the induction process and to identify the inducers and their regulation.

Pectate lyase synthesis is also regulated by catabolite repression (Hugouvieux-Cotte-Pattat *et al.*, 1986) as well as environmental conditions (Perombelon, 1990). Gene fusion studies have shown that all genes of the pectin catabolic pathway are under co-ordinate negative regulation via a repressor (KdgR) (Condemine and Robert-Baudouy, 1987b). Analysis of regulatory regions of several of these genes reveals a highly conserved motif of dyad symmetry that may correspond to the KdgR-binding site

(Reverchon *et al.*, 1989). These observations suggest that pectate lyases are an important disease factor, but they do not mean that these enzymes are all that is needed for pathogenesis. Although pectate lyases have been shown to be induced by catabolites of GA or dGA, these molecules must first enter the bacterial cell for intracellular catabolism to occur. Studies on the uptake of these molecules have been sparse and limited to glucuronic acid (Hugouvieux-Cotte-Pattat *et al.*, 1983), galacturonic acid (San Francisco and Keenan, 1993; Freeman and San Francisco, 1994), and 2-keto-3-deoxygluconate (Condemine and Robert-Baudouy,1987).

Erwinia chrysanthemi transport systems

To date, two transport systems have been characterized in *Erwinia chrysanthemi* Ec16 in the uptake products of extracellular pectin degradation. Recently, the *exuT*-complementing DNA of *E. chrysanthemi* EC16 was cloned (Freeman and San Francisco, 1994) and sequenced (Freeman and San Francisco, unpublished). The *exuT* DNA encodes proteins that transport galacturonic acid across the cell membrane to be utilized in catabolism. The *exuT* transport system is probably controlled by the negative regulatory element *exuR* (Hugouvieux-Cotte-Pattat *et al.*, 1983). GA uptake is inducible, with the addition of GA, dGA, or a mixture of pectin and PGA into the growth medium increasing activity two- to threefold. Cyanide and 2,4-dinitrophenol inhibited uptake activity 85 to 90%, respectively, suggesting that GA uptake is energy dependent (San Francisco and Keenan, 1993). Experimental studies suggest that expression of the *exuT* gene(s) is under the control of pectin derivitives- GA, dGA, PGA or a metabolite of the molecules (San Francisco and Keenan, 1993; Freeman and San Francisco, 1994).

The *kdgT* permease transports ketodeoxyuronates, which are: DKI, DKII, and KDG. Expression of *kdgT* is induced by polygalacturonate, galacturonate, unsaturated digalacturonate, and gluconate, but only weakly by DKI and DKII, and not at all by

external KDG (Allen *et al.*, 1983). *kdgT* belongs with *kdgK* and *kdgA* to the *kdg* regulon (Pouyssegur and Lagarde, 1973). The gene is under the control of the *kdgR* gene product, a global negative regulatory element for the polygalacturonate degradation pathway, which has been postulated to regulate the expression of *kduD, kduI, kdgA, kdgK, olg, pme,* and the five isozymes of pectate lyase (Condemine and Robert-Baudouy, 1987a). A sequence corresponding to the KdgR-binding site has been identified in the regulatory regions of all the genes controlled by *kdgR*. An oligonucleotide corresponding to the *kdgT* operator binds *in vitro* and *in vivo* the KdgR protein (Nasser *et al.*, 1994). Although KdgT is responsible for the transport of KDG into the cell, it is unable to grow on this substrate as the sole carbon and energy source (Pouyssegur, 1974). KDG can enter into the bacterium only when *kdgT* in mutant cells is constitutively expressed because its affinity for this transport system is low (Condemine and Robert-Baudouy, 1987a). The failure to grow on KDG is not the result of a lack of a transport system since mutants exhibiting derepressed synthesis of this protein could easily be found and KDG could be accumulated above the external concentration in galacturonate-induced strains. The absence of growth can be attributed to two factors: low affinity of KDG for its own permease and low basal level of the permease (Condemine and Robert-Baudouy, 1987a). KdgT also mediates the entry of DKI and DKII which are competitive inhibitors of KDG uptake (Condemine and Robert-Baudouy, 1987). The KDG transport system should be called the ketodeoxyuronate transport system. The *kdgT* permease gene has been sequenced and shows strong homology with the *E. coli kdgT* gene. The hydrophobic character (63% nonpolar amino acid sequence) of the *kdgT* permease indicates that it is probably an intrinsic membrane transport system (Allen *et al.*, 1989). The *kdgT* permease is dependent upon the proton motive force, as demonstrated by the sensitivity of KDG uptake to the uncoupler carbonyl cyanide m-chlorophenyl hydrazone. This dependence suggests that the system consists of

a single component, since the coupling of energy to the specific transport of its substrate occurs within a single functional protein (Allen *et al.*, 1989).

San Francisco and Keenan (1993) investigated whether or not the *exuT* gene product could facilitate the uptake of dGA. *E. coli* carrying an R' plasmid capable of complementing an *exuT* mutant in *E. chrysanthemi* B374, can grow on both GA and its radioactive analog. However, it is unable to transport or utilize dGA, on which *E. chrysanthemi* can readily grow. *E. chrysanthemi* mutants have been identified that can take up monomers and trimers of GA but are unable to transport dGA (dimer) (Xiang and San Francisco, unpublished). These preliminary results suggest that a separate transporter is necessary for the uptake of these dimer molecules.

Cloning of the *exuT* DNA

GA is transported into the cytoplasm by the *exuT* DNA product(s). Using an *E. chrysanthemi* genomic library in cosmid pLARF3, a 3.4-kb fragment of the *E. chrysanthemi* EC16 chromosome capable of complementing both growth and uptake of an *E. chrysanthemi* mutant was identified and cloned (Freeman and San Francisco, 1994). The 3.4-kb EcoRV fragment carrying the *exuT* DNA was subcloned into pUC19 and designated pTTU-1 (Figure 4). Tn*phoA* gene fusions with the cloned DNA indicated two regions are required for complementation of the *exuT* mutations in *E. chrysanthemi* ERH215, and the resulting alkaline phosphatase hybrid polypeptides provided for the localization of at least one gene product to the membrane of the cell (Freeman and San Francisco, 1994). Sequencing of the 3.4-kb fragment revealed the presence of 2 open reading frames (San Francisco *et al.*, unpublished data).

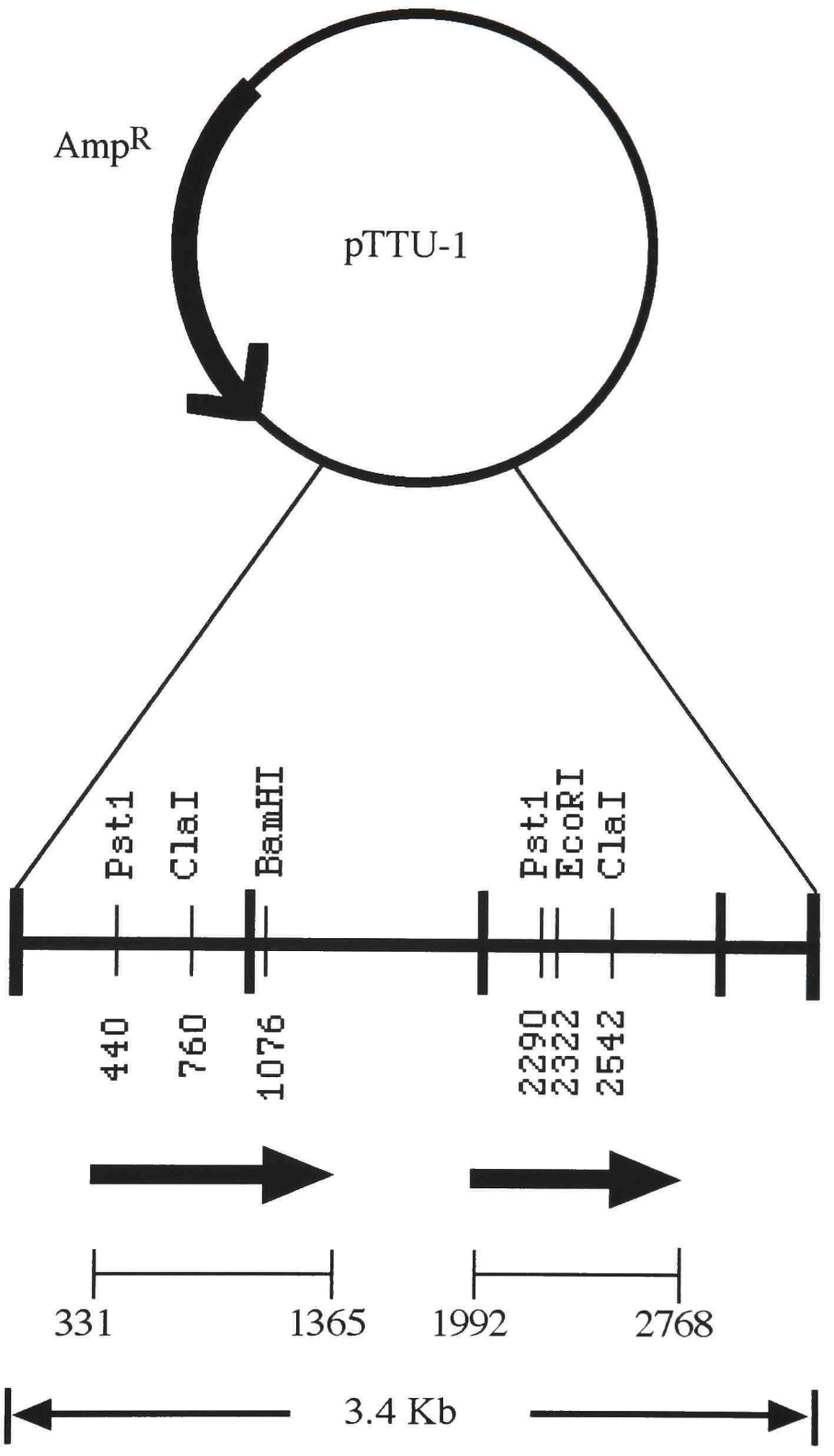

Figure 4. Restriction map and open reading frames of pTTU-1. Line art illustrating the cloning of the 3.4-Kb *exuT* complementing DNA into the SmaI site of pUC19. The bold arrows indicate the location and direction of the two open reading frames.

CHAPTER II

GENERATION OF DIRECTED AND UNMARKED CHROMOSOMAL DELETIONS USING MARKER EXCHANGE-EVICTION MUTAGENESIS

Introduction

Site-directed mutagenesis by marker exchange of a mutated cloned gene (Ruvkun and Ausubel, 1981) provides a method for generating specific mutations that are difficult to screen. This technique allows for a mutation to be directed to a specific locus on the bacterial chromosome by homologous exchange recombination of an insertionally inactivated cloned gene with the functional allele. Generated mutants can then be analyzed to determine how the genes function in relation to pathogenicity. However, the presence of antibiotic resistance markers can result in limitations in complementation analysis or in the construction of strains with multiple mutations (Reid and Collmer, 1987). In order to generate multiple gene mutations it is necessary to generate unmarked yet targeted mutations. Figure 5 demonstrates a marker-exchange-eviction mutagenesis method based on a double cross-over event between the chromosome and the manipulated DNA as described by Reid and Collmer (1987). The process requires the insertion of a *nptI-sacB-sacR* cartridge that encodes both kanamycin resistance and sucrose sensitivity (Figure 6). The *sacB* gene encodes levansucrase, an enzyme that is secreted by *Bacillus subtilus* (Gay *et al.*, 1983). In Gram-negative bacteria, in the presence of 5% sucrose, the enzyme is secreted into the periplasmic space and becomes lethal to the cell. *sacR* is a regulatory element for *sacB* (Steinmetz *et al.*, 1985). Both parts of the cartridge are used in the construction of the unmarked chromosomal mutation. After the cartridge has been inserted into the DNA of interest, chromosomal exchange occurs using low phosphate medium. The low phosphate results in the loss of the unstable replicon inside the cell. This is a

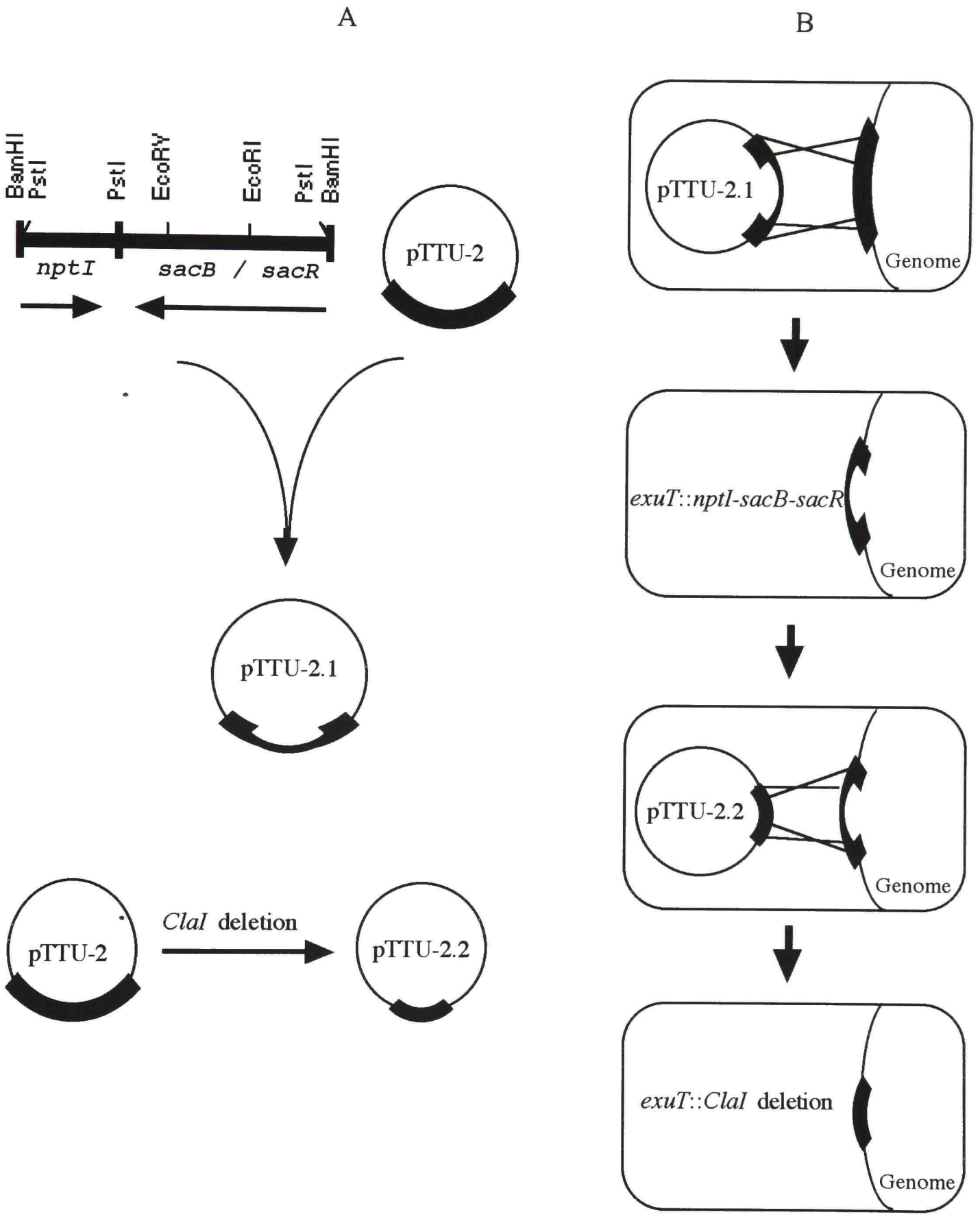

Figure 5. Marker exchange-eviction mutagenesis of the *exuT* DNA. Panel A shows the *in vitro* steps. The *exuT* gene is inactivated by the insertion of the cartridge to yield pTTU-2.1. Plasmid pTTU2.2 was generated by *ClaI* digestion of pTTU-2, followed by self-ligation. Panel B demonstrates the in vivo steps. An *nptI-sacB-sacR*-marked *exuT* mutation was introduced into the genome by exchange recombination resulting in kanamycin resistance and sucrose sensitivity. The cartridge was evicted by introducing pTTU2.2 and selecting for a second exchange on the basis of sucrose tolerance. The resulting mutant was ExuT$^-$, Kan^S, and contained a 1.7-kb deletion in the *exuT* gene.

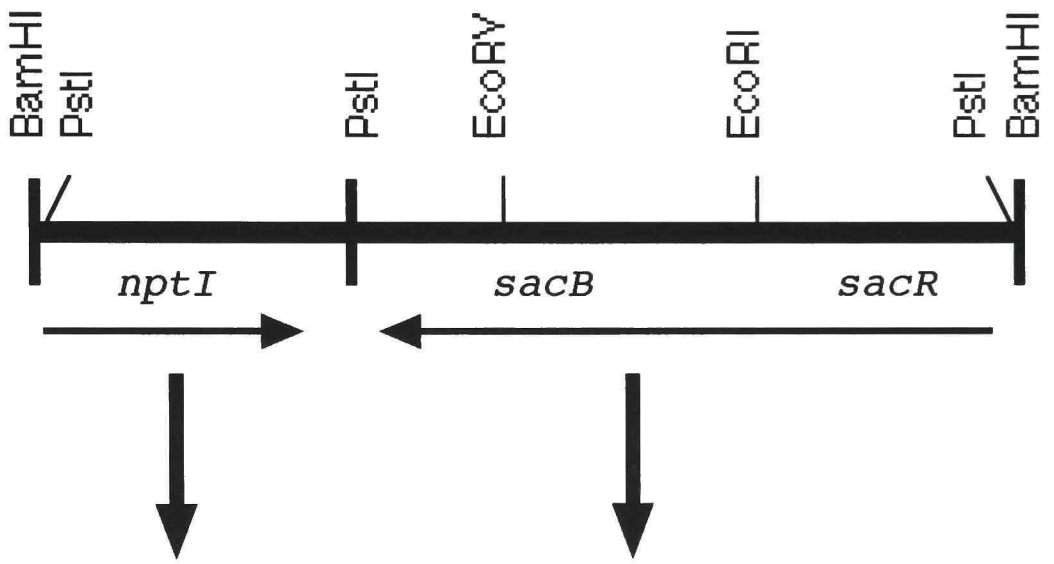

Figure 6. *nptI-sacB-sacR* mutagenizing cassette. The *nptI-sacB-sacR* cartridge is contained on a 3.8 kb BamHI fragment. The cartridge was used to generate *exuT* mutants in *E. chrysanthemi* EC16.

result of limited phosphate to maintain DNA structures. This process selects for homologous exchange between the chromosome and the mutant gene(s) carried on the unstable plasmid with the chromosome. Kanamycin resistance is used in the first step to select for the exchange of the marked gene mutation with the chromosome. Sucrose sensitivity is used to select for the eviction of the marked gene mutation with the unmarked gene mutation. This process has been used numerous times to generate targeted, unmarked chromosomal mutations (Roeder and Collmer, 1985; Reid and Collmer, 1987, 1988; He and Collmer, 1990; Kamoun *et al.*, 1992).

Materials and Methods

Bacterial strains and plasmids.

Strains and plasmids that were used to carry out this study are listed in Table 2.

Media and growth conditions

E. chrysanthemi EC16 and CUCPB0873 was used to generate targeted unmarked deletions in the GA uptake genes on the bacterial chromosome. *Erwinia* and *E. coli* strains were grown in Luria-Bertani medium (Miller, 1972) except where noted. *Erwinia* and *E. coli* strains were grown shaken at 30°C and 37°C, respectively. Basal salts media (low phosphate) was used to select for the marker exchange mutagenesis (Torriani, 1960). Growth rates were determined by using M9 broth (Miller, 1972) supplemented with 0.1% GA as the sole carbon source. Pectate semisolid agar (Starr *et al.*, 1977) was used to determine the pectolytic ability of the generated mutants. King's media (King *et al.*, 1954) was used to grow the strains before Belgian endive and potato infections. When indicated, antibiotics were used in the growth media at the following concentrations: ampicillin (50μg/ml), kanamycin (50μg/ml), and tetracycline (10μg/ml).

Table 2
Strains and plasmids used in this study.

Designation	Relevant Characteristic	Origin(reference)
Escherichia coli		
HB101	*leu pro*A2 *thi rec*A13 *hsd*S20	Boyer
HB101:pRKR013	Mating helper strain	A. Chatterjee
E. chrysanthemi		
EC16	Wild-type	A. Chatterjee
CUCPB0873	Wild-type	A. Chatterjee
Plasmids		
pRK415	Tet^r	Ditta
pTTU1	pUC19 carrying *exuT* from EC16	Freeman and San Francisco
pTTU2	pRK415 carrying *exuT* from EC16	This study
pTTU2.1	pTTU2 derivative with a ClaI deletion in exuT containing *nptI-sacB-sacR* cartridge from pUM24, Tet^r, Kan^r	This study
pTTU2.2	pTTU2 derivative with ClaI deletion in *exuT*, Tet^r	This study
pUM24	pUC4K derivative containing *nptI-sacB-sacR* cartridge, Amp^r,Kan^r	Reid and Collmer

DNA manipulations

Plasmid DNA was isolated either by alkaline lysis (Sambrook *et al.*, 1989) or QIAprep-spin Plasmid Kit (QIAGEN). DNA restriction modifications, ligations, and agarose gel electrophoresis were performed according to Sambrook *et al.* (1989). *E. coli* was routinely transformed by the calcium chloride method (Hanahan *et al.*, 1983). *E. chrysanthemi* strains were transformed by either electroporation or bacterial mating (Van Haute *et al.*, 1983; Roeder and Collmer, 1985) Electroporation was performed according to manufacturer's instructions using a BRL Cell Porator, Voltage Booster and micro-electroporation chambers at 330 μF, 4 kW, and 12.5 kV/cm.

Plasmid construction for marker exchange mutagenesis of the *exuT* DNA of *E. chrysanthemi*

Marker exchange-eviction mutagenesis was carried out using the *nptI-sacB-sacR* cartridge essentially described by Reid and Collmer (1985) (Figure 5). Plasmid pTTU-1 was digested with the restriction enzymes *Sac*I and *Hind*III and electrophoresed in a 0.7% agarose gel. The gel fragments of 3.4-kb *exuT* DNA and the full-length 10.5-kb pRK415 (Ditta *et al.*, 1985) derivative were excised and purified using Gene Clean Kit (BIO101). The 3.4-kb *exuT* DNA fragment was then subcloned in the low-copy number plasmid pRK415. Ligation of the *exuT* DNA was performed according to Sambrook *et al.* (1989). The pTTU-2 recombinant plasmid was then transformed into *E. coli* HB101 by the calcium chloride procedure (Hanahan *et al.*, 1983) and the cells were spread on LB agar containing tetracycline. pTTU-2 was digested with the restriction enzyme *Cla*I, deleting a 1.7-kb DNA fragment to disrupt both open reading frames. Plasmid pUM24 was digested with *Bam*HI, blunt-ended with Klenow (Sambrook *et al.*, 1989), and electrophoresed in a 0.7% agarose gel. The 3.8-kb fragment was excised, purified by the use of the Gene Clean Kit and ligated into pTTU-2 (Figure 7). pTTU-2.1 was transformed into HB101 and spread on LB agar containing tetracycline and kanamycin. Plasmid DNA from these transformants

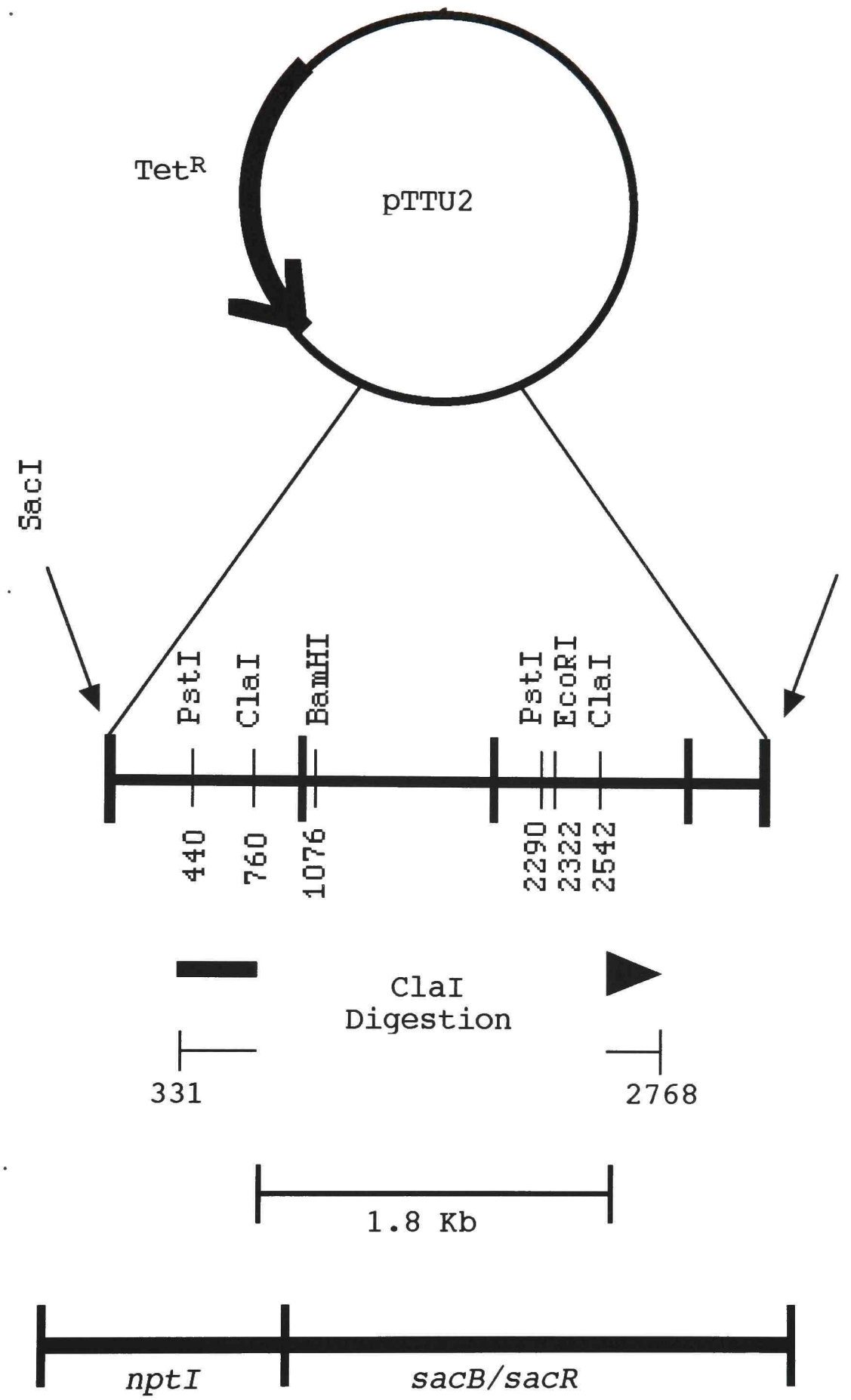

Figure 7. Construction of pTTU-2.1. pTTU-2 was generated from subcloning the *exuT* DNA into the *sacI/HindIII* mutiple cloning site of pRK415. pTTU-2.1 was subsequently generated by blunt-end ligation of the *nptI-sacB-sacR* cartridge into the *ClaI* deletion site of the *exuT* DNA. Both ORFs have now been disrupted.

was isolated and restriction-mapped (Figure 8). Plasmid pTTU-2.1 retained all of the parent sequences and contained the *nptI-sacB-sacR* cartridge within the *exuT* DNA. pTTU-2.1 was electroporated into *E. chrysanthemi* and plated onto LB agar containing tetracycline and kanamycin.

Selection for exchange recombination

E. chrysanthemi EC16:pTTU2.1 was grown in basal medium with 0.1% glycerol, 0.250 μM potassium phosphate, 0.02% Casamino acids and kanamycin at 30°C with shaking for 3 days. The culture was then serial diluted and spread on LB agar containing kanamycin. Isolated colonies were then replica-plated on LB-tetracycline and LB-kanamycin plates to screen for tetracycline-sensitive isolates, which would indicate loss of the plasmid.

Eviction of the kanamycin marker in the *exuT* mutant

Plasmid pTTU-2.2 was constructed from pTTU-2 by restriction enzyme digestion with *Cla*I followed by self-ligation and selection for tetracycline resistance HB101 transformants. Restriction analysis confirmed the *Cla*I deletion (Figure 9). pTTU2.2 was electroporated into *E. chrysanthemi* EC16 and spread onto LB agar containing tetracycline and kanamycin. To select for the second exchange an isolated colony was inoculated into LB broth, grown at 30°C for 30 hours and plated on LB agar containing 5% sucrose. The colonies that were sucrose-tolerant were then replica-plated onto kanamycin plates and tetracycline plates. The phenotypic characteristics of kanamycin- and tetracycline-sensitive and sucrose-tolerant EC16 colonies indicated that an unmarked, chromosomal mutation in the *exuT* DNA had been generated.

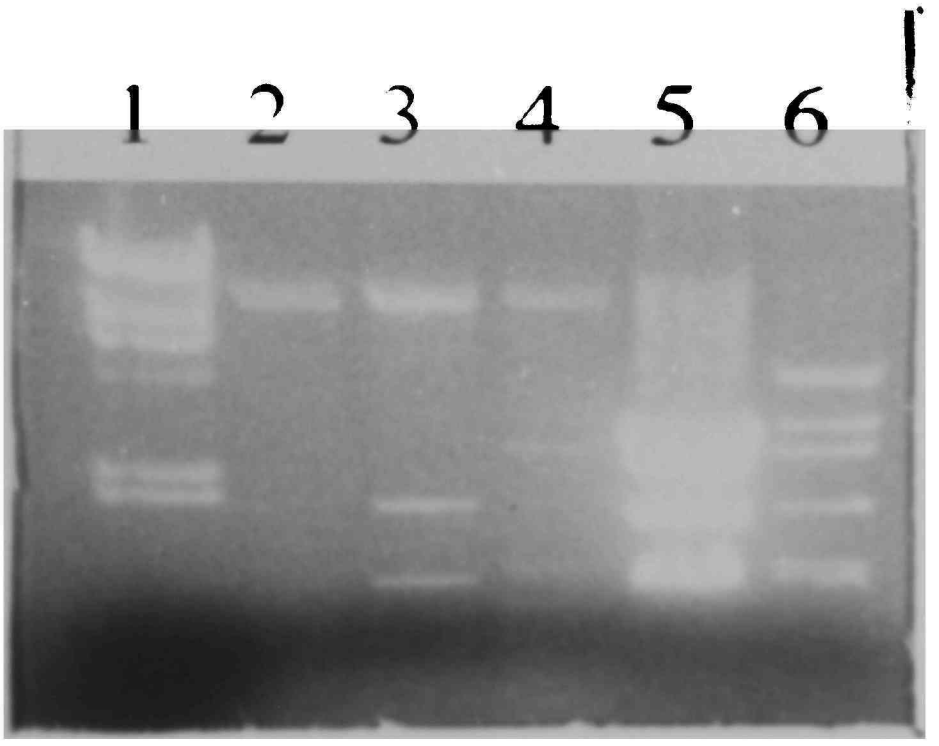

Figure 8. Restriction analysis of pTTU-2.1. *Pst*I digests of plasmid DNA resolved by electrophoresis through a 0.7% agarose gel. Lanes: 1, 1 *Hind*III; 2, pRK415; 3, pTTU-2; 4, pTTU-2.1; 5, pTTU-1; 6, pUM24. Restiction analysis reveals that pTTU2 contains the *exuT* DNA subcloned from pTTU-1. pTTU-2.1 constructed from a *Cla*I deletion of pTTU-2 contains the *nptI-sacB-sacR* cartridge disrupting both open reading frames.

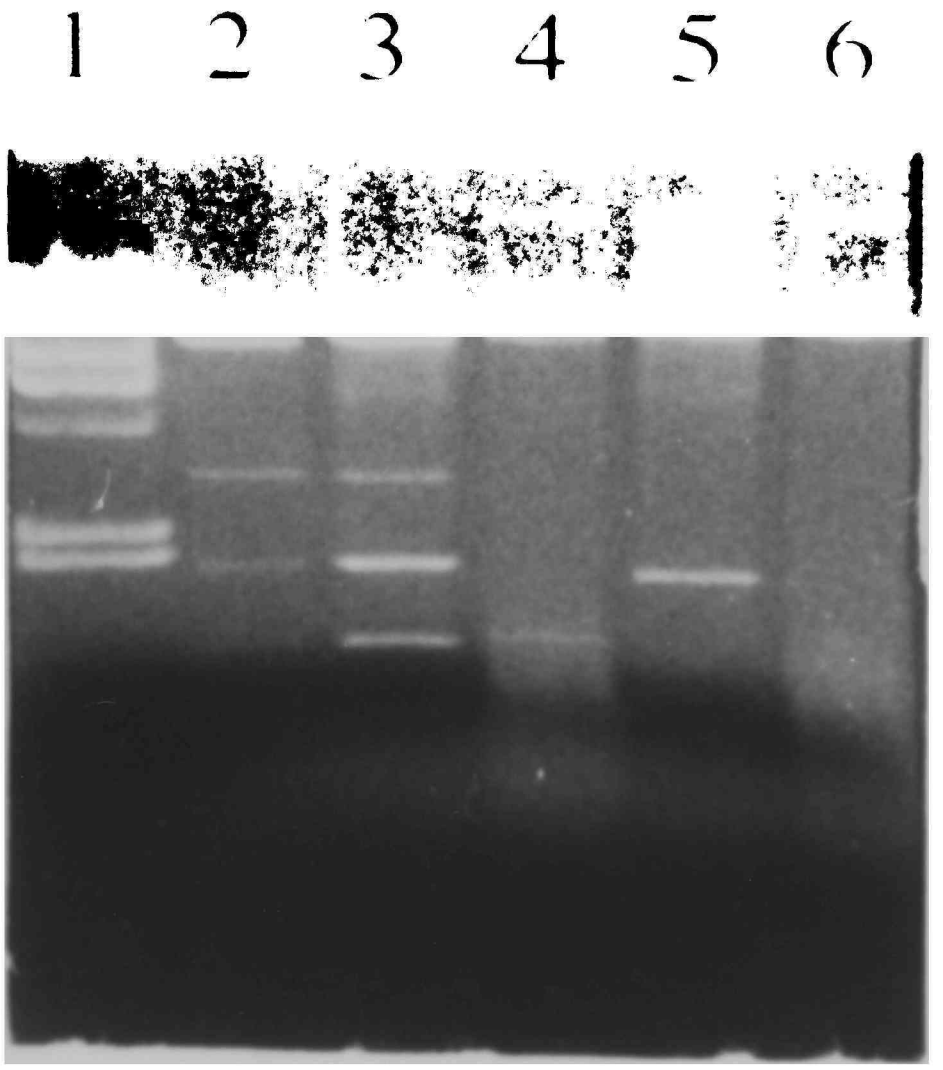

Figure 9. Restriction analysis of pTTU-2.2. Digests of plasmid DNA resolved by electrophoresis through a 0.7% agarose gel. Lanes 2-4 were digested with *Pst*I and lanes 5 and 6 were digested with *Cla*I: 1, λ *Hind*III; 2, pRK415 ; 3, pTTU-2; 4, pTTU-2.2; 5, pTTU-2; 6, pTTU-2.2. Restiction analysis reveals that pTTU-2 contains the *exuT* DNA subcloned from pTTU-1. Lane 6 shows that pTTU-2.2 contains the *Cla*I deletion of the *exuT* DNA.

Bacterial matings

Transformants of *E. coli* HB101 containing either pTTU2.1 or pTTU2.2 were grown in LB broth containing kanamycin and tetracycline or tetracycline, respectively. HB101:pRKR013 helper strain was grown up in liquid LB containing kanamycin. *Erwinia* strains were grown in liquid LB without antibiotics. Overnight cultures (1 ml) were centifuged and washed three times and resuspended in 1 ml of 10 mM $MgSO_4$. One hundred microliters of *E. chrysanthemi* was spotted onto LB agar without antibiotics followed by 30 μL each of donor and helper cells. The mating mixture was incubated at 37°C for 8-16 hours, then the lawn of cells was resuspended in 10 mM $MgSO_4$ and spread onto M9 minimal plates containing 0.1% pectin/PGA and either kanamycin and tetracycline or tetracycline alone to select for plasmid mobilization into *E. chrysanthemi*. *E. chrysanthemi* CUCPB0873 minimal plates contained 0.02% Casamino acids. Selection was confirmed by pitting on pectate semi-solid agar plates (Starr *et al.*, 1977).

Measurement of growth rates

Growth rates were monitored using Klett flasks containing 15 ml of M9 minimal media containing either GA or glycerol at a final concentration of 0.1%. The flasks were inoculated with overnight cultures to equivalent initial optical densities (approximately 1:30 dilution) 10. The cultures were then shaken in a water bath at 30°C. Absorbances were measured at hourly intervals using a Klett-Summerson colorimeter with a green filter.

Galacturonic acid uptake assay

GA uptake was measured with the use of a radio-labeled analog, ^{14}C-GA (Sigma Chemical Co.) as described by San Francisco and Keenan, (1993). Cells were grown under inducing and non-inducing conditions in M9 media containing GA or glycerol. Cells in the early log phase were washed in transport buffer (M9 salts, 5 mM $MgSO_4$, and 0.3

mM dithiothreitol), and starved for 15 minutes. ^{14}C-GA (58 mCi/mmol) was added to the assay to a final concentration of 0.1 mM. The final assay volume of 0.5 ml contained 50 μL of cell suspension. The cell suspension was incubated for 2 min at the assay temperature (30°C), prior to the addition of the radioactive substrate. After the labeled substrate was added, aliquots were removed at specific time intervals and uptake terminated by filtration through a 0.45 μm nitrocellulose filter, and washed with 2 mls of prewarmed transport assay buffer. The filters were then dried, and the uptake of ^{14}C-GA was quantified in a liquid scintillation counter (Beckman 7600).

Potato tuber virulence assay

Store-bought Russet potatoes were washed with sterile water, surfac-sterilized with 0.01% bleach for 1 minute with gentle rubbing, and rinsed well with sterile water (modification of Maher and Kelman 1983). Potatoes were then cut into 20-mm slices and placed in sterile glass Petri dishes. Inoculum (10^8 cells/ml) was obtained from heavily streaked King's media plates and serial diluted to determine concentrations. Disposable pipette tips containing 100μl of cells resuspended in M9 minimal media were streaked onto the potato surface. The Petri dishes were then incubated at 30°C for 48 hours. Maceration of potato tubers were monitored on the basis of color, and the softness of the darkened tissue.

Belgian endive infections

Belgian endive (whitloof chicory) leaves were inoculated at small wounds with 5 x 10^5 cells of either wild-type *Erwinia* or the generated mutants. The wounds were made by using a sterile toothpick to gently scratch the leaf's surface (Bauer *et al*, 1994). Inoculum was obtained from heavily streaked King's media plates resuspended in M9, and serially diluted to determine concentrations. Cells were pipetted into the wound and the leaves

were placed into glass petri dishes. Kimwipes dampened with water were placed in the bottom of the dish to provide humidity. The Petri dishes were incubated at 30°C for 48-72 hours. Maceration of the leaves was monitored on the basis of color, and the softness of the darkened tissue.

Results and Discussion

Initial construction of the directed unmarked mutations in *E. chrysanthemi* were carried out utilizing the vector pBR322 (Boliva *et al.*, 1977). pBR322 is a moderate-copy number plasmid. Due to various transformation problems and plasmid instability in *E. chrysanthemi*, the vector pRK415 was selected to carry out the marker exchange-eviction mutagenesis. pRK415 is derived from the naturally occuring plasmid RK2 (Ditta *et al.*, 1985). High-efficiency mobilization of pRK415 from *E. coli* to other bacterial hosts is accomplished with the assistance of a self-transmissible helper plasmid, pRKRO13. pRK415 is quite unstable in the absence of selection, making it an ideal plasmid for marker exchange-eviction mutagenesis. This plasmid contains the pUC19 multiple cloning site and carries the tetracycline resistance gene which is used for selection of the lost plasmid after the exchange.

The *exuT* DNA required for complete complementation of an *exuT* mutant was subcloned into pRK415 to generate pTTU-2. DNA sequence analysis and DNA expression utilizing the T7 RNA polymerase/promoter expression system revealed two open reading frames (Melkus *et al.*, 1995 in preparation). These open reading frames were inactivated by the deletion of the *Cla*I fragment and the insertion of the *nptI-sacB-sacR* cartridge generating pTTU-2.1 (Figure 7). pTTU-2.1 was transformed into *E. chrysanthemi* EC16. The transformants were tetracycline-resistant, kanamycin-resistant and sucrose-sensitive. After selection for the first exchange, colonies with the phenotypic traits of tetracycline sensitivity, kanamycin resistance and sucrose sensitivity indicated that

homologous exchange recombination occurred between the inactivated DNA and the chromosome with the loss of the plasmid (Figure 6). In order to study the effects of the mutation on plant tissue, the second step of evicting the cartridge was necessary (since the presence of the cartridge would make the cells sensitive to sucrose, which is formed in plant tissue, unpublished data).

pTTU-2.2 was mated into the *exuT* deletion strains and selected for the second homologous exchange. Colonies that displayed tetracycline sensitivity, kanamycin sensitivity, and sucrose tolerance were selected. Whole cells from four possible *exuT* mutants were analyzed by SDS-PAGE to verify that they were *E. chrysanthemi* and not *E. coli* HB101 from the bacterial mating (Figure 10). Comparison of the wild type EC16 with the generated mutants to identify the *exuT* proteins was not able to be visualized. This is due to membrane proteins being poorly expressed. Many membrane protein DNA sequences have weak Shine-Delgarno sequences and/or transcriptional destabilizing stem and loop formation.

To test whether the targeted *exuT* mutation had any effect on the ability of the bacteria to grow on GA as a sole carbon source, a growth curve analysis was conducted. Figure 11 shows 4 such mutants and their ability to grow on GA. Mutants 1.1 and 1.2 were not markedly different from the parent strain EC16. Mutants 7.1 and 7.2, however, showed a distinct lag period over the first 4-9 hours, suggesting some limitation to growth on GA. Transport analysis using ^{14}C-GA were carried out to observe if the lesion was indeed in the transport apparatus. Figure 12 shows uptake data for mutants 7.1 and 7.2 and the parent strain EC16. At a substrate concentration of 0.1 mM, there was a slight, although distinct difference in uptake of the radioactive analog. ^{14}C-GA was also measured in mutants 1.1 and 1.2, which closely resembled the parent EC16 uptake levels. The complete loss of GA uptake activity was not expected because the *kdgT* system has been shown to also take up GA (Condemine and Robert-Baudouy, 1987). To initially

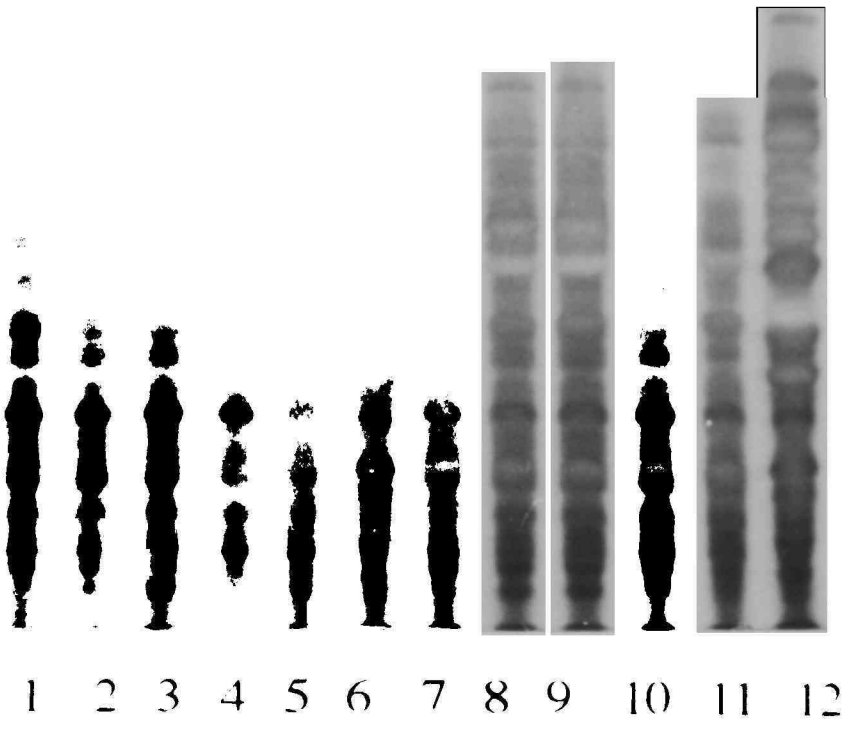

Figure 10. SDS PAGE of EC16 and EC16 E*xuT*⁻ mutants. Whole cells were electrophoresed on 10% SDS-PAGE in the following order: lane 1, EC16; 2-5 *exuT* mutants, 1.1, 1.2, 7.1, and 7.2; 6 *E.coli* HB101; lanes 7-12 are the samples repeated. This gel demonstrates that the mutants are *E. chrysanthemi* and not contaminants of HB101 from the mating.

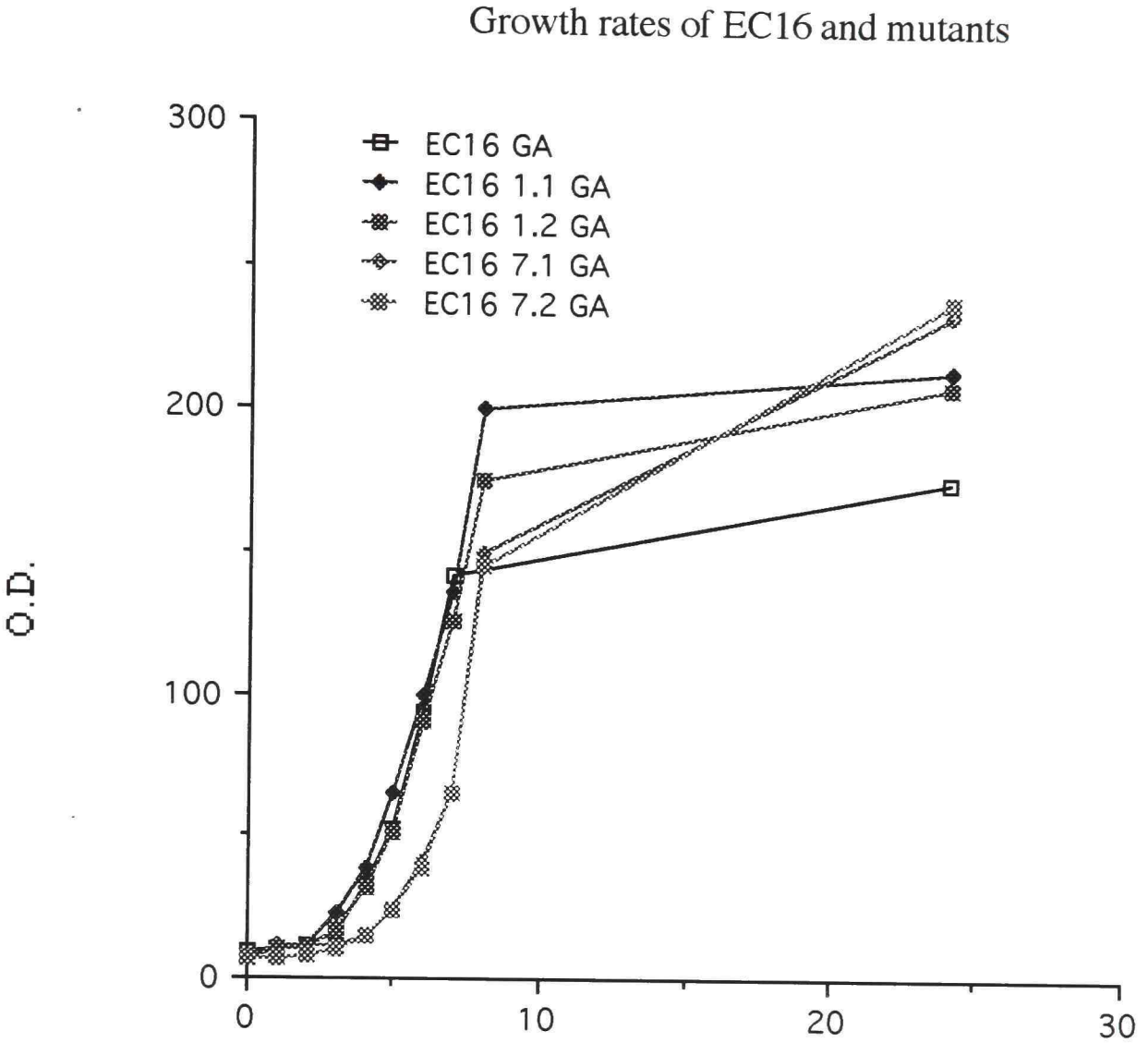

Figure 11. Growth rates of EC16 and EC16 ExuT^{-}. Cultures were grown up overnight in 0.1% pectin/PGA. These cultures were then used to inoculate Klett flasks containing 0.1% GA as the sole carbon source and incubated at 30°C.

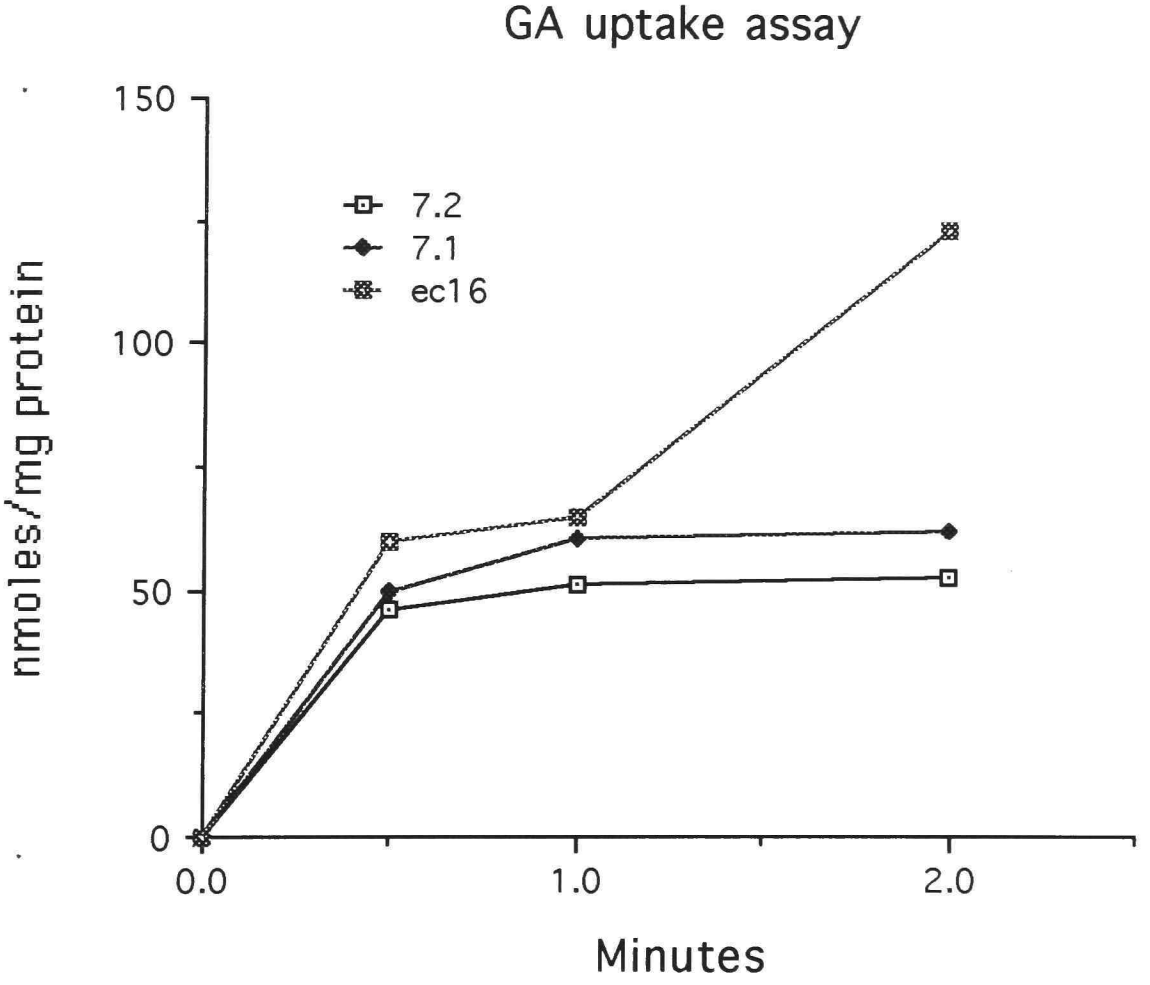

Figure 12. Uptake of ^{14}C-GA by EC16 and EC16 ExuT.$^{-}$

assess the effect of *exuT* transport mutants on the disease-causing potential of *E. chrysanthemi,* the ability to produce pectate lyases and polygalacturonic acid hydrolases were tested on pectate semi-solid agar. EC16 and each of the mutants spotted onto pectate semi-solid agar were able to cause pitting in the medium. Pitting occurs due to the secretion of pectate lyases into the medium, breaking down pectin into simpler constituents. This result was expected and confirmed that the GA monomer is inferior to either of the dimers as an inducer of pectate lyases (Collmer and Bateman, 1981).

The ability of pectate lyase- and polygalacturonic acid-producing strains of *E. chrysanthemi* to macerate potato-tuber tissue has been well documented (Collmer and Keen, 1986). Potato tubers were sliced and inoculated with 10^8 cells and then incubated for 48 hours at 30°C. Maceration of tissue and spread of infection was monitored based on color, size of the darkened area and softness of darkened tissue. Figure 13 shows the *exuT* mutants and the wild type EC16. Mutants 1.1 and 1.2 had a reduced area of infection and symptoms. Mutant 1.2 did have a small region of softening representative of the parent strain. Mutants 7.1 and 7.2 showed increased infection area with characteristic darkening and softening of soft-rot, slightly greater than the parental strain.

E. chrysanthemi EC16 and generated mutants were inoculated into a scratched leaf of Belgian endive and incubated for 48 hours (Figure 14). Leaves were infected with both wild type and a mutant to compare tissue maceration. Mutant 1.1 demonstrated a phenotype very characteristic of EC16. The infection sizes, color, and maceration area were approximately equal. Mutant 1.2 revealed a reduced disease state compared to the parent's infection area in the Belgian endive leaf. Both mutants 7.1 and 7.2 had comparable infection areas and softening to each other. These mutants had a much greater infection area, darkening, and softening than the EC16 wild type. These mutants demonstrated systemic disease symptoms in the leaf characterized by their vast area of infection. The severity and unique phenotypes of disease result from virulence

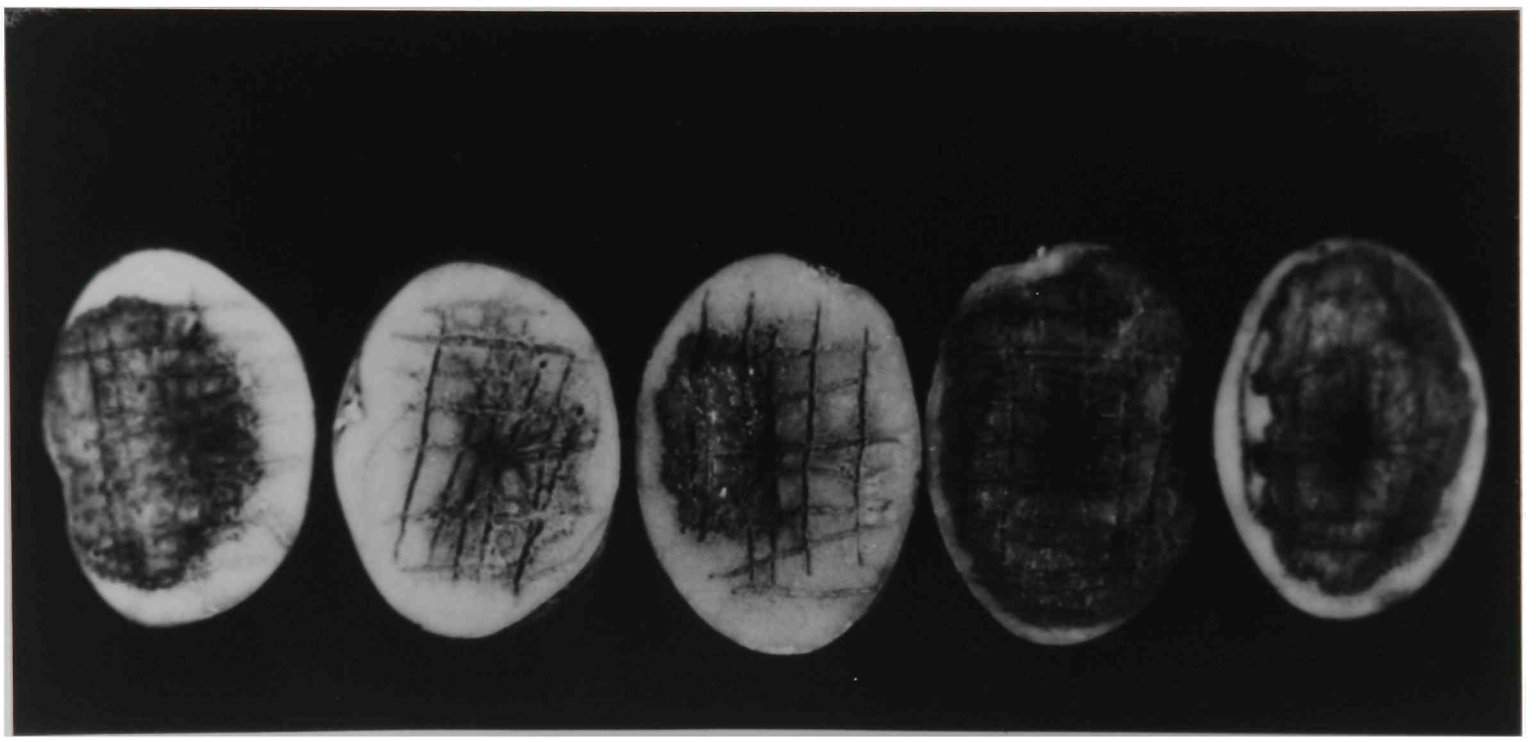

Figure 13. Potato tuber virulence assay. Potato tuber slices were inoculated with *E. chrysanthemi* or the *exuT*$^-$ mutants and incubated for 48 hours at 30°C. Comparison of the mutants and the wild type *E. chrysanthemi* reveals that mutants 1.1 and 1.2 have a decreased ability to cause tissue maceration. Mutants 7.1 and 7.2 appear to have an increased maceration ability.

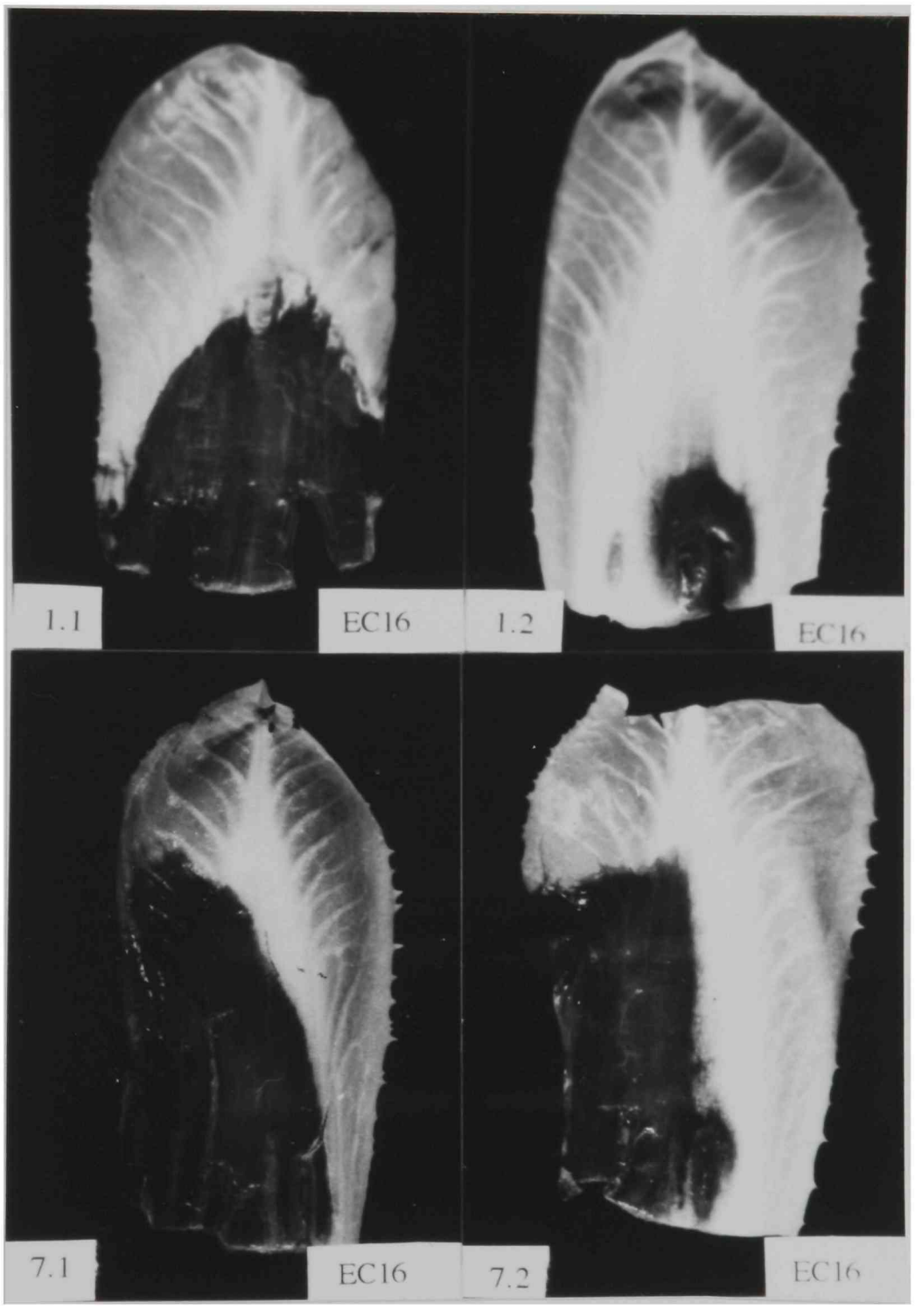

Figure 14. Belgian endive infections. *E. chrysanthemi* EC16 and generated mutants were inoculated into a scratched leaf and incubated for 48 hours. Leaves were infected with both wild type and mutant to compare tissue maceration.

determinants encoded by the bacterial genes controlling functions such as plant cell wall-degrading enzymes, extracellular polysaccharides, hormones, and toxins (Long *et al.*, 1993). The virulence determinants could be accessory functions for metabolic pathways. Some regulatory genes, including members of the two-component regulatory family, have coordinated effects on diverse virulence functions. These include the secretion of hydrolytic enzymes, carbohydrate uptake, and toxin production (Winans, 1991). The VirA and VirG genes of *Agrobacterium tumefaciens* represent the prototype two-component regulatory system. VirG protein is the direct regulatory protein that binds upstream of *vir* promotors following phosphorylation by VirA. VirA is a membrane-spanning protein and is the environmental sensor (Long *et al.*, 1993).

The two-component system could be a likely model for the GA uptake system. DNA analysis has revealed two open reading frames, one of which is a membrane protein based on the hydrophobicity and transposon-fusion analysis. The two genes have been designated as *exuTA* and *exuTB*. From the sequence analysis, ExuTA is the membrane-spanning protein and could act as the sensor and ExuTB could be a negative regulator. The *exuT* transport system has been hypothesized to be under the control of the negative regulator *exuR* (Hugouviex-Cotte-Pattat *et al.*, 1983). ExuR has not yet been identified. By disrupting both of these proteins in *E. chrysanthemi* the bacterial cell could be derepressed for certain virulence functions, allowing for the typical virulence phenotype expressed in the 7.1 and 7.2 mutants. Further studies are needed to identify the role of each of these proteins. Complementation assays will allow for the assessment of ExuTA and ExuTB in GA uptake. It is possible that complex environmental signals, such as GA or other chemical inducers, will be as important in determining the virulence phenotype of *E. chrysanthemi* as they are in the *Rhizobium*- and *Agrobacterium*-plant associations.

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