Calcium-Dependent Pectate Lyase Production in the Soft-Rotting Bacterium *Pseudomonas fluorescens*

C.-H. Liao, D. E. McCallus, and J. M. Wells

Eastern Regional Research Center, Agricultural Research Services, U.S. Department of Agriculture, 600 East Mermaid Lane, Philadelphia, PA 19118.

Corresponding author: C.-H. Liao, telephone 215-233-6471.

Reference to a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Accepted for publication 11 March 1993.

ABSTRACT

Liao, C.-H., McCallus, D. E., and Wells, J. M. 1993. Calcium-dependent pectate lyase production in the soft-rotting bacterium *Pseudomonas fluorescens*. Phytopathology 83:813-818.

Pectate lyase (PL) is the principal or sole enzyme responsible for maceration of plant tissue caused by most strains of soft-rotting pseudomonads. Production of PL in four out of 25 *Pseudomonas fluorescens* (or *P. marginalis*) strains examined was not induced by the enzyme substrate, polygalacturonate (PGA), but was induced by Ca^{2+}. These four strains produced 10 times more PL in medium containing 1 mM $CaCl_2$ than in one containing no $CaCl_2$ supplement. Over 86% of total PL produced by these strains in $CaCl_2$-supplemented medium was excreted into the culture fluid. Only a small portion (13%) of total PL produced by these strains in $CaCl_2$-deficient medium was detected in the extracellular fraction. Ca^{2+} thus affected not only the amount but also the final destination of PL produced by these pseudomonads. Additionally, all four strains were unable to use PGA as a nutritional source when cultured in Ca^{2+}-deficient medium, which indicates that the initial step of PGA degradation was mediated by Ca^{2+}-dependent PL and not by Ca^{2+}-independent polygalacturonase. The optimal Ca^{2+} concentration required for PL production in one of these strains, CY091, was determined to be 0.2 mM. A linear correlation was observed between the amounts of PL produced and the concentrations of Ca^{2+} included in the medium. Furthermore, the requirement of Ca^{2+} for PL induction could be replaced by Sr^{2+} but not by other divalent cations, such as Zn^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, and Ba^{2+}. Because of the indispensable role of Ca^{2+} in PGA degradation and in PL production, the possibility of using the ion-chelating agent ethylenediaminetetraacetic acid (EDTA) for control of Pseudomonas rot was evaluated. EDTA exhibited bactericidal activity against *P. fluorescens* at a minimal inhibitory concentration of 4 mM. When assayed on potato tuber disks, EDTA at a concentration of 0.13 mM (40 ppm), which is 30-fold lower than the minimal inhibitory concentration, was effective in preventing *P. fluorescens* from growing and causing maceration in potato tuber tissue.

Additional keywords: enzyme export, potato rot control.

Pectolytic, fluorescent pseudomonads, mainly strains of *Pseudomonas fluorescens* (or *P. marginalis*) and *P. viridiflava*, account for over 40% of bacterial rot of fruits and vegetables in storage and during transit (15). These pseudomonads are unique among postharvest pathogens in that they are able to grow under refrigerated conditions and to use a wide variety of simple compounds as carbon (C) and energy sources (1,15). Both *P. fluorescens* and *P. viridiflava* have been shown to cause diseases of plants in the field (1,12). However, they appear to be more often associated with decay of plant products after harvest (12). Because of the psychrophilic nature of the bacteria, refrigeration currently employed to prolong the shelf life and to reduce microbial decays of fresh produce is ineffective in suppressing rot caused by *P. fluorescens* and *P. viridiflava.* No control measure specifically targeted against this group of soft-rotting pathogens is presently available, but such measures might be developed when more is learned about the biochemical and genetic mechanism by which these pathogens cause diseases in plants.

The ability of *P. fluorescens* and *P. viridiflava* to induce maceration of plant tissues is primarily due to their ability to produce pectolytic enzymes that are capable of degrading pectic components in plant cell walls. Although a few strains of *P. fluorescens* have been shown to produce polygalacturonase (21,33), pectin methylesterase (21), and pectin lyase (28,29), almost all strains of soft-rotting pseudomonads so far examined produce pectate lyase (PL) (5,7,10,21,35). Recently, we investigated the isoelectric focusing profiles of PL samples produced by 10 strains of *P. fluorescens* and eight strains of *P. viridiflava* (10) and found that the pectic enzyme system of these strains was much simpler than that of *Erwinia* (10). Unlike the multiple PL isozymes system (pI 4.5–10.0) in *Erwinia* (2), all *P. fluorescens* and *P. viridiflava* strains examined produced a single alkaline PL with approximate isoelectric points of pI 9.7 and 10.0, respectively (10). Results from genetic studies with transposon mutagenesis (12) and gene cloning (11,14) indicate that the alkaline PL produced by *P. fluorescens* and *P. viridiflava* is the principal or sole enzyme responsible for maceration of plant tissues.

At present, very little is known about the biochemical mechanism governing PL production in soft-rotting pseudomonads. Production of PL in the majority of *P. fluorescens* strains appears to be induced by pectic substrates (5,7,21,33) or by plant tissue extracts (33,35). However, in some strains, PL production is not affected by the type of C source included in the medium (21,35). Recently, we investigated the mode of PL production in an unusual strain of *P. fluorescens* designated CY091 (11). We found that PL production in this strain, although not affected by the type of C source included in the medium, appeared to be induced by Ca^{2+}. In this study, we surveyed the regulation of PL production in 24 other strains of *P. fluorescens* (15). We identified three additional strains that produced PL in a mode similar to that observed in strain CY091. Furthermore, we report here that the effect of Ca^{2+} on PL production in strain CY091 is dose-dependent and that Ca^{2+} is replaceable by Sr^{2+}. Previously, we suggested that control of Pseudomonas rot might be achieved by manipulating free Ca^{2+} available in plant tissue (11). Here, we report that the ion-chelating agent ethylenediaminetetraacetic acid (EDTA), at an extremely low concentration (0.13 mM), is sufficient to inhibit maceration of potato tuber tissue caused by *P. fluorescens*.

MATERIALS AND METHODS

Bacterial strains and culture media. Twenty-five soft-rotting strains of *P. fluorescens* previously isolated and characterized in our laboratory (15) were used. After initial screening, four strains (CY091, BC-05-1B, PJ-08-30, and LU-04-2B), which produced high levels of PL in media containing glucose, glycerol, or polygalacturonate (PGA), were identified and chosen for further studies. Strain CY091, which had been previously used to clone the PL gene and to demonstrate the Ca^{2+} dependency for PL production (11), was used to determine other parameters affecting enzyme production. A kanamycin-resistant derivative of strain CY091 (designated CY091B) was obtained by transposon mutagenesis as described elsewhere (16). Strain CY091B was prototrophic and showed no alteration in tissue-macerating ability. This strain was used to evaluate the efficacy of EDTA as a disease control agent in potato tuber assays (to be described later).

Media that were used during the study included 1) *Pseudomonas* agar F (Difco Laboratories, Detroit, MI), 2) Luria broth (GIBCO/BRL Laboratories, Grand Island, NY), and 3) minimal salt (MS) standard medium (pH 7.1) containing 0.7% K_2HPO_4, 0.2% KH_2PO_2, 0.02% $MgSO_4{\cdot}7H_2O$, 0.1% $(NH_4)_2SO_4$, 1 mM $CaCl_2$, and 0.4% glucose, glycerol, or PGA (grade 1, no. P-3899, Sigma Chemical Co., St. Louis, MO). When required, $CaCl_2$ in the standard MS medium was omitted or adjusted to various concentrations ranging from 0.01 to 1.00 mM. All cultures were incubated at 26 C and were shaken (120 rpm) when liquid media were used. The initial inoculum density in liquid media was in the range of 3–5 × 10^5 cells per milliliter, and incubation lasted 60 h unless otherwise indicated.

Enzyme assays. Polygalacturonase (3) and PL (12) activities were determined in accordance with methods previously described. One unit of PL activity was defined as the amount of enzyme that caused an increase of 1.0 absorbance unit per minute at 232 nm and 20 C. The reaction was carried out in a 0.5-ml volume containing 100 mM Tris HCl (pH 8.0), 1 mM $CaCl_2$, 0.2% PGA, and enzyme sample. The specific enzyme activity was calculated and expressed as units of PL activity per 10^{10} cells. Cell numbers were estimated from optical density (OD600) readings; a sample with an OD600 of 1.5 was assumed to contain 10^9 cells per milliliter. Activities of PL in cultures or in subcellular fractions were determined according to the methods reported previously (11).

Effects of C sources, divalent salts, and EDTA on PL production. Bacterial strains were grown in MS media containing various types and concentrations of C sources, divalent salts, and EDTA to determine 1) the effects of C sources and Ca^{2+} on PL production and 2) the optimal concentration and the dependency of Ca^{2+} for PL induction. Three C sources examined were glucose, glycerol, and PGA, and nine divalent salts tested were $CaCl_2$, $BaCl_2$, $FeSO_4$, $MnCl_2$, $MgCl_2$, $SrCl_2$, $ZnCl_2$, $NiCl_2$, and $CuCl_2$. After incubation, total PL activities in cultures and partial enyzme activities in subcellular fractions were analyzed in accordance with methods previously described (11). Briefly, cells were separated from the culture medium by centrifugation (10,000 *g* for 10 min), and the clear supernatant was assayed for extracellular activity. The cell pellet was washed and resuspended in 50 mM Tris HCl (pH 7.2), and cells in the suspension were then disrupted by ultrasonication. After that, cell debris was removed by centrifugation (25,000 *g* for 20 min), and the clear supernatant was assayed for cell-bound activity. Occasionally, cells were disrupted by adding 1/200 volume of toluene to the culture or the cell suspension to release the cell-bound enzyme.

To determine whether the requirement for Ca^{2+} in PL production is dependent on the concentration of Ca^{2+}, strain CY091 was grown in the MS-glycerol medium containing various concentrations (0.01–3.00 mM) of $CaCl_2$. After incubation, total PL activities in the cultures were measured. To further demonstrate that PL production is inducible by Ca^{2+}, two experiments were conducted. In the first experiment, strain CY091 was grown for 24 h in the MS-glycerol medium without the addition of $CaCl_2$. After that, $CaCl_2$ was added to the culture to a final concentration of 0.2 mM, and incubation continued for 36 h. In the second experiment, strain CY091 was grown for 24 h in the MS-glycerol medium with the addition of 0.2 mM of $CaCl_2$. Then EDTA at a sublethal level (0.5 mM) was added to the culture, and incubation continued for another 36 h. Both PL activities and cell populations in the culture were monitored at 12-h intervals for 60 h.

Inhibitory effect of EDTA on bacterial growth and tissue maceration. Luria broth (20 ml) with or without 2 mM $CaCl_2$ was inoculated with cells of strain CY091B grown overnight to an initial cell density of approximately 10^5 cells per milliliter. The inoculated medium was then equally dispensed into a series of 10 tubes. Filter-sterilized EDTA stock solution (0.5 M) was added to tube 1 to a final concentration of 16 mM, and serial twofold dilutions of EDTA were made in tubes 2–9. Tube 10, which contained no EDTA, was used as a control. After incubation at 26 C with shaking (120 rpm) for 60 h, bacterial growth was recorded as indicated by an increase in turbidity in the tube. The minimal inhibitory concentration was defined as the minimal concentration of EDTA that was capable of preventing the increase of cell density in cultures. Similarly, 20 ml of sterile water with or without 2 mM $CaCl_2$ was inoculated with cells of strain CY091B grown overnight to 10^5 cells per milliliter. The suspension of bacteria in water was then equally dispensed in a series of 10 tubes (16 × 125 mm). EDTA was again added to the first tube to a final concentration of 16 mM and was serially diluted twofold in tubes 2–9. A surface-sterilized potato tuber disk, 8 × 6 × 3 mm, prepared as previously described (15), was placed in each tube. After incubation at 26 C with shaking for 60 h, each tube was vigorously vortexed for 1 min. The development of soft rot, as indicated by total disintegration of potato tuber tissue, was determined and recorded. To avoid the growth of bacterial contaminants, kanamycin was added to each tube at a final concentration of 10 μg/ml.

RESULTS

Ca^{2+} requirement for pectin utilization. Twenty-one of the 25 *P. fluorescens* strains examined in the study produced PL at levels four- to 70-fold higher in the medium containing PGA than in the medium containing glucose or glycerol. The presence of 1 mM $CaCl_2$ in the MS medium was essential for these 21 strains to produce high levels of PL and to use PGA as the sole C source for growth. Four of the 25 strains examined produced nearly equally high levels of PL in the medium containing either glucose, glycerol, or PGA (Table 1). Analysis of variance showed that production of PL by these four strains (CY091, BC-05-1B, PJ-08-30, and LU-04-2B) was not significantly ($P \geq 0.05$) affected by the type of C source included in the medium. All four strains were unable to use PGA or pectin when cultured in the MS medium without the addition of 1 mM $CaCl_2$. However, when glucose or glycerol was included as the C source, the growth of these four strains in the MS media with or without 1 mM $CaCl_2$ was not significantly different ($P \leq 0.01$). The inability of these pseudomonads to grow in the MS-PGA medium lacking 1 mM $CaCl_2$ was therefore not due to the Ca^{2+} deficiency. In addition, polygalacturonase activity was not detected in culture filtrates prepared from any of these four strains. The action of Ca^{2+}-dependent PL, but not of Ca^{2+}-independent polygalacturo-

TABLE 1. Effect of carbon sources on pectate lyase production in four soft-rotting strains of *Pseudomonas fluorescens*

	Total activity (units/10^{10} cells)[x,y,z]		
Strain	Glucose	Glycerol	Polygalacturonate
CY091	35.1 ± 4.2	41.8 ± 6.5	69.2 ± 3.1
BC-05-1B	28.6 ± 6.3	25.7 ± 3.9	47.2 ± 2.8
PJ-08-30	71.4 ± 5.3	80.6 ± 7.3	120.3 ± 6.7
LU-04-2B	90.5 ± 3.7	110.8 ± 11.3	88.5 ± 8.3

[x] One unit of activity was defined as the amount of enzyme that caused an increase of 1.0 absorbance unit per minute at 232 nm and 20 C.

[y] Grown in minimal salt media (see Materials and Methods) containing 1 mM $CaCl_2$ and 0.4% glucose, glycerol, or polygalacturonate.

[z] Values are the mean of three separate experiments plus or minus the standard error. Analysis of variance indicated no significant ($P \geq 0.05$) effect of carbon source on pectate lyase production.

TABLE 2. Effect of $CaCl_2$ on pectate lyase production in four strains of *Pseudomonas fluorescens*[x]

Strain	Without $CaCl_2$			With $CaCl_2$		
	Total activity[y,z] (units/10^{10} cells)	Location (%)		Total activity (units/10^{10} cells)	Location (%)	
		Extracellular	Cell-bound		Extracellular	Cell-bound
CY091	4.8 ± 0.9	11	89	37.8 ± 5.8	93	7
BC-05-1B	2.5 ± 0.7	8	92	31.2 ± 3.2	86	14
PJ-08-30	19.1 ± 3.1	4	96	82.8 ± 6.1	91	9
LU-04-2B	4.7 ± 1.2	13	87	93.4 ± 2.8	89	11

[x] Grown in minimal salt medium supplemented with glycerol (0.4%) (see Materials and Methods) and containing or lacking 1 mM $CaCl_2$.
[y] One unit of activity was defined as the amount of enzyme that caused an increase of 1.0 absorbance unit per minute at 232 nm and 20 C.
[z] Values are the mean of three separate experiments plus or minus the standard error. Analysis of variance indicated significant ($P < 0.01$) effect of $CaCl_2$ on enzyme production. The enzyme activities that were cell-bound in cultures with or without $CaCl_2$ were not signficantly different at $P = 0.05$.

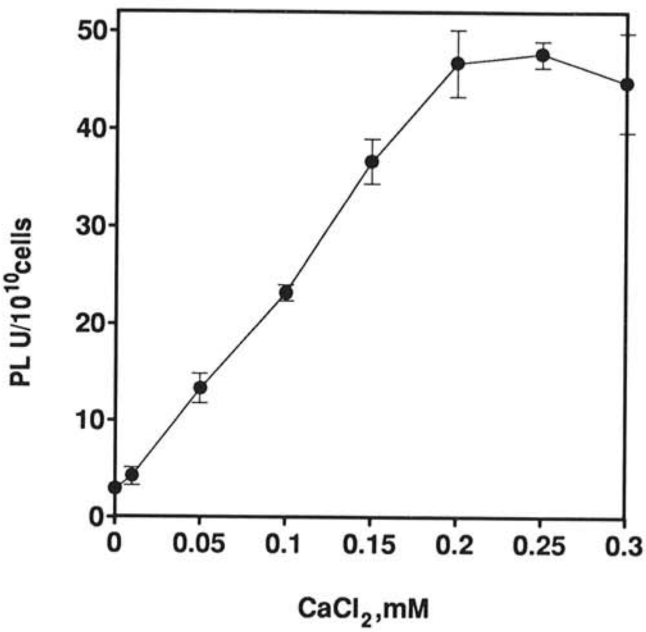

Fig. 1. Effect of various concentrations of $CaCl_2$ on pectate lyase production in *Pseudomonas fluorescens* strain CY091. The bacterium was grown in minimal salt medium supplemented with glycerol (0.4%) and various concentrations of $CaCl_2$. Values are the mean of three independent experiments, and brackets indicate the standard error. One unit (U) of activity was defined as the amount of enzyme that caused an increase of 1.0 absorbance unit per minute at 232 nm and 20 C.

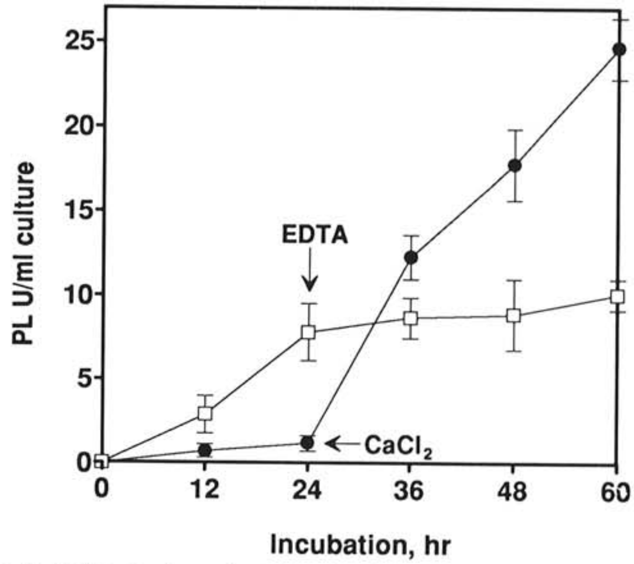

Fig. 2. Ca^{2+} induction and ethylenediaminetetraacetic acid (EDTA) inhibition of pectate lyase production in *Pseudomonas fluorescens* strain CY091. The bacterium was first grown in minimal salt medium supplemented with glycerol (0.4%) and containing $CaCl_2$ (□) or not including $CaCl_2$ (●) for 24 h. Then, 0.5 mM EDTA or 0.2 mM $CaCl_2$ was added to the culture, and incubation continued for another 36 h. Values are the mean of three independent experiments, and brackets represent the standard error. One unit (U) of activity was defined as the amount of enzyme that caused an increase of 1.0 absorbance unit per minute at 232 nm and 20 C.

nase activity, was required by these strains for degradation and utilization of pectin or PGA.

Ca^{2+} requirement for PL production. To study the effect of Ca^{2+} on PL production, strains CY091, BC-05-1B, PJ-08-30, and LU-04-2B were grown in the MS-glycerol medium with or without the addition of 1 mM $CaCl_2$. In the absence of 1 mM $CaCl_2$, all four strains produced very small amounts of PL and retained a major proportion (over 87%) of the PL within the cells (Table 2). In the presence of 1 mM $CaCl_2$, the four strains produced four to 20 times more PL than that detected in Ca^{2+}-deficient medium. Moreover, at least 86% of the total PL produced by these strains in $CaCl_2$-containing medium was detected in the culture fluid. The Ca^{2+} content in the medium thus affected not only the amount but also the destination of PL synthesized. Furthermore, when strain CY091 was grown in the MS-glycerol media containing 0.01–3.00 mM $CaCl_2$, a linear relationship was observed between the amount of PL produced and the concentration of $CaCl_2$ included in the medium (Fig. 1). The highest level of PL produced by strain CY091 was detected in the medium containing 0.20 mM $CaCl_2$. No significant increase in PL activity was detected in media containing higher concentrations (0.30–3.00 mM) of $CaCl_2$.

To further demonstrate that PL production in strain CY091 was indeed inducible by Ca^{2+}, two additional experiments were conducted. Results showed that only a slight increase in PL activity occurred in the first 24 h when $CaCl_2$ was absent, but a sharp increase in PL activity followed immediately after the addition of $CaCl_2$ (Fig. 2). Similarly, when strain CY091 was initially grown in the medium containing 0.2 mM $CaCl_2$, PL activities increased steadily in the first 24 h when free Ca^{2+} was readily available but leveled off immediately following the addition of EDTA. Final populations of strain CY091 grown under these conditions were similar and fell within the range of 4–8 × 10^9 cfu/ml. The change in the pattern of PL production as shown in Figure 2 was therefore not due to the difference in growth rate following the addition of $CaCl_2$ or EDTA.

To determine whether other divalent cations could be substituted for Ca^{2+} in PL induction, strain CY091 was grown in the MS-glycerol media containing one of nine divalent salts. Strain CY091 did not grow in the medium containing $NiCl_2$ or $CuCl_2$ at a concentration as low as 0.05 mM, which indicates that both Cu^{2+} and Ni^{2+} are highly toxic to *P. fluorescens*. However, strain CY091 did grow equally well in the medium containing 0.2 mM of $CaCl_2$, $BaCl_2$, $ZnCl_2$, $FeSO_4$, $MgCl_2$, $SrCl_2$, or $MnCl_2$. The amount of PL produced by strain CY091 in the medium containing $CaCl_2$ or $SrCl_2$ was about 10-fold higher than that produced by this bacterium in the medium containing $BaCl_2$, $FeSO_4$, $MnCl_2$, $MgCl_2$, or $ZnCl_2$ or in the medium containing none of these nine divalent salts (Table 3). Furthermore, over 90% of total PL produced by strain CY091 in the medium containing $CaCl_2$ (or $SrCl_2$) was excreted into the culture fluid. In contrast, a large proportion of PL produced by this strain in the media containing other divalent salts was retained within the cells. Also, Fe^{2+} appeared to have an adverse effect on PL production. Strain CY091 produced approximately 75% less PL in the medium containing 0.2 mM $FeSO_4$ than in that containing no $FeSO_4$ (Table 3).

Inhibitory effect of EDTA on bacterial growth and on tissue maceration. *P. fluorescens* was highly susceptible to the bactericidal activity of EDTA. The minimal inhibitory concentration of EDTA required for inhibiting the growth of strain CY091B in Luria broth was determined to be 4 mM (Table 4). When this rich broth was supplemented with 2 mM $CaCl_2$, the amount of EDTA required for inhibition of bacterial growth remained about the same (in the range of 4–8 mM). This indicates that the bactericidal activity of EDTA is not simply due to its Ca^{2+}-chelating property. When assayed on potato tuber disks, EDTA at a concentration of 0.13 mM (approximately 40 ppm) was sufficient to prevent strain CY091B from growing and causing maceration in potato tuber tissue. However, when potato tuber disks were submerged in water supplemented with 2 mM $CaCl_2$, the EDTA concentration required for prevention of tissue maceration in potato tuber disks increased to 4 mM, the level that was required for inhibition of bacterial growth in rich broth. Although the bactericidal activity of EDTA in rich medium could not be overcome by the addition of $CaCl_2$, the inhibitory effect of EDTA on soft rot development could be reversed by adding 2 mM $CaCl_2$.

DISCUSSION

The data presented here and elsewhere (11) demonstrate that Ca^{2+} is an important factor in the regulation of PL production in *P. fluorescens*. In pectin-inducible strains, Ca^{2+} as an enzyme cofactor is involved in the initial steps of pectin degradation required by the bacteria to generate unsaturated oligouronates needed for PL induction (2). In pectin-noninducible strains (such as the four strains identified in this study), Ca^{2+} appears to act as an environmental factor regulating PL synthesis and/or PL export in the bacteria. Since PL synthesis and PL export are two independent events, it is presently unclear whether the effect of Ca^{2+} on PL production occurs in transcription, translation, or protein secretion. Previously, it was shown that Ca^{2+} is essential for maintaining the structural integrity and functional activity of proteases produced by *P. fluorescens* strains associated with raw milk spoilage (19). We also found that heat stability of PL is greatly enhanced in the presence of Ca^{2+} or other positively charged molecules such as polylysine (L.-J. Wong and C.-H. Liao, *unpublished*). The possibility that the lower PL activities detected in Ca^{2+}-deficient media (Table 2) may result from formation of inactive PL needs to be further investigated. Recently, we found that protease production in strain CY091 is also regulated by Ca^{2+} (16). Moreover, we found that partially purified proteases from strain CY091 were unable to digest PL proteins (16). These results imply that lower PL activities detected in Ca^{2+}-deficient media (Table 2) are probably not due to degradation of PL.

Divalent cations have been shown to affect extracellular enzyme production in two other microbial systems. McQueen-Ross et al (20) reported that esterase production in *Streptomyces scabies* was inducible by Zn^{2+}. Reverchon et al (25) showed that *Escherichia coli* cells carrying *Erwinia* PL genes produced higher levels of PL in a medium containing 0.05–0.50 mM $CaCl_2$. Since neither of these studies included experiments to determine the location of the enzyme in the subcellular fractions, it is not known whether the decrease in enzyme activity was due to a defect in the enzyme synthesis or to enzyme export. In this study, we found that over 87% of total PL produced by four *P. fluorescens* strains grown in Ca^{2+}-deficient media remained cell-bound. It is possible that the outer membrane of *P. fluorescens* grown under Ca^{2+}-deficient conditions may form an incompetent configuration (8) unsuitable for protein translocation. Recently, we identified and cloned a genomic DNA fragment (designated *rep*) from *P. viridiflava*, which appears to contain a cluster of genes required for PL, protease, and exopolysaccharide (alginate) production (13). The exact function of *rep* genes and its relation to the Ca^{2+} effect on PL production as presented in this paper are presently obscure. We suspect that Ca^{2+} may serve as an environmental signal, which is directly or indirectly involved in the activation of *rep* genes required for the synthesis and/or export of extracellular enzymes and exopolysaccharide.

The presence of Ca^{2+} in the pectic polysaccharide matrix of plant cell walls is essential for maintaining the strength and flexibility of the walls (24). A high Ca^{2+} content in plant tissue has been shown to reduce internal breakdown of pectic polysaccharides in plant cell walls (9) and to inhibit the polygalacturonase activities of plants (9) and pathogens (4,23,32). Based on these findings, it has been suggested that the disease resistance mechanism of plants may be enhanced by raising the Ca^{2+} content in plant tissue. So far, this approach has proven useful for controlling pre- and postharvest diseases caused by polygalacturonase-pro-

TABLE 3. Effect of divalent ions on pectate lyase production in *Pseudomonas fluorescens* strain CY091[x]

Ion	Total activity[y,z] (units/10^{10} cells)	Location (%) Extracellular	Cell-bound
Basal level	3.1 ± 0.5 a	16	84
Ca^{2+}	39.5 ± 2.1 b	92	8
Sr^{2+}	33.4 ± 3.8 b	89	11
Mg^{2+}	6.2 ± 1.2 c	68	32
Ba^{2+}	3.5 ± 0.2 c	21	79
Fe^{2+}	0.8 ± 0.4 c	3	97
Mn^{2+}	3.7 ± 0.5 c	58	42
Zn^{2+}	4.5 ± 0.7 c	12	88

[x] Grown in minimal salt medium supplemented with glycerol (0.4%) containing 0.2 mM $CaCl_2$, $BaCl_2$, $FeSO_4$, $MnCl_2$, $MgCl_2$, $SrCl_2$, or $ZnCl_2$. The activity in the medium containing none of the above divalent salts was considered the basal level.

[y] One unit of activity was defined as the amount of enzyme that caused an increase of 1.0 absorbance unit per minute at 232 nm and 20 C.

[z] Values are the mean of three separate experiments plus or minus the standard error. Means followed by the same letter within the column were not significantly different at $P = 0.05$.

TABLE 4. Inhibitory effect of ethylenediaminetetraacetic acid (EDTA) on growth on *Pseudomonas fluorescens* strain CY091B in culture medium and on development of soft rot in potato tuber disks

	EDTA concentration (mM) 8	4	2	1	0.5	0.25	0.13	0.07	0
Bacterial growth in Luria broth[y]									
Without 2 mM $CaCL_2$	−	−	+	+	+	+	+	+	+
With 2 mM $CaCl_2$	−	+	+	+	+	+	+	+	+
Development of soft rot in potato tuber disks[z]									
Without 2 mM $CaCl_2$	−	−	−	−	−	−	−	+	+
With 2 mM $CaCl_2$	−	−	+	+	+	+	+	+	+

[y] + = Growth; − = no growth.

[z] Soft rot development was indicated by total disintegration of potato tuber tissue. + = Soft rot development detected; − = soft rot development not detected.

ducing fungal pathogens, such as *Penicillium expansum* (4) and *Botrytis cinerea* (4,32). It is not totally certain, however, whether the same approach can be employed to control diseases caused by PL-producing pathogens, such as *Erwinia* and *Pseudomonas.* McGuire and Kelman (17,18) previously reported that potato tubers with high Ca^{2+} content were more resistant to Erwinia rot. However, a recent study by Tzeng et al (31) showed that the Ca^{2+} content alone cannot entirely account for resistance or susceptibility of a specific potato cultivar to *Erwinia.* Pagel and Heitefuss (22) also found a slight correlation between the Ca^{2+} content and the resistance of potato cultivars to bacterial rot. For pathogens that produce PL as the principal disease factor, the presence of readily available Ca^{2+} in the plant environment apparently favors PL production. Pagel and Heitefuss (23) showed that the presence of 0.05–0.50 mM $CaCl_2$ in potato tubers infected with *Erwinia* stimulates PL activities and increases the rate of tissue maceration. The results (Table 4) presented in this study show that 1) the presence of free Ca^{2+} in plant tissue is essential for the development of Pseudomonas rot, 2) prevention of Pseudomonas rot can be achieved by treating potato tuber disks with the Ca^{2+} chelator EDTA, and 3) the suppressive effect of EDTA on soft rot development can be reversed by adding 2 mM $CaCl_2$. Previously, it was shown that the presence of Ca^{2+} at levels higher than the PL activity optimum (0.5 mM) reduces the release of neutral sugars from potato cell walls (27) and slightly inhibits PL activities in potato tubers infected with *Erwinia* (23). There is no conclusive evidence, however, that total inhibition of PL activities and cell wall degradation can be achieved by raising the Ca^{2+} content to a level that is technically and commercially feasible (4).

In this study, we demonstrated that the addition of the ion-chelating agent EDTA at a low concentration (0.13 mM) is sufficient to prevent *P. fluorescens* from growing and causing tissue maceration in potato tuber disks. The effectiveness of EDTA as a disease control agent is mainly due to its bactericidal activity and its ability to bind Ca^{2+} required for PL activity and for bacterial growth in plants. The bactericidal effect of EDTA was previously demonstrated with *P. aeruginosa* (6) and *P. fluorescens* (34). Despite the finding that EDTA may be potentially useful for control of Pseudomonas rot, the possibility of undesirable effects that may arise from tTA EDTA treatment need to be considered. The Ca^{2+} deficiency in plant tissue as a result of the prolonged EDTA treatment may cause release of pectic fragments from cell walls (26), stimulate plant and microbial polygalacturonase activities (9,22,23), and interfere with plant physiological functions related to senescence and disease resistance (30). To avoid all these undesirable effects, a treatment method that does not require a prolonged EDTA exposure needs to be devised.

LITERATURE CITED

1. Brocklehurst, T. F., and Lund, B. M. 1981. Properties of pseudomonads causing spoilage of vegetables stored at low temperature. J. Appl. Bacteriol. 50:259-266.
2. Chatterjee, A. K., and Vidaver, A. K. 1986. Genetics of pathogenicity factors: Application to phytopathogenic bacteria. Pages 122-149 in: Advances in Plant Pathology. Vol. 4. D. S. Ingram and P. H. Williams, eds. Academic Press, London.
3. Collmer, A., Ried, J. L., and Mount, M. S. 1988. Assay methods for pectic enzymes. Methods Enzymol. 161:329-335.
4. Conway, W. S., Sams, C. E., McGuire, R. G., and Kelman, A. 1992. Calcium treatment of apples and potatoes to reduce postharvest decay. Plant Dis. 76:329-334.
5. Fuchs, A. 1965. The *trans*-eliminative breakdown of Na-polygalacturonate by *Pseudomonas fluorescens.* Antonie van Leeuwenhoek J. Microbiol. Serol. 31:323-340.
6. Gray, G. W., and Wilkinson, S. G. 1964. The action of ethylenediamine tetraacetic acid on *Pseudomonas aeruginosa.* J. Appl. Bacteriol. 28:153-164.
7. Hagar, S. S., and McIntyre, G. A. 1972. Pectic enzyme produced by *Pseudomonas fluorescens,* an organism associated with "pink-eye" disease of potato tubers. Can. J. Bot. 50:2479-2488.
8. Kojima, M., Suda, S., Hotta, S., and Hamada, K. 1969. Induction of pleomorphy and calcium ion deficiency in *Lactobacillus bifidus.* J. Bacteriol. 102:217-220.
9. Konno, H., Yamaya, T., Yamasaki, Y., and Matsumoto, H. 1984. Pectic polysaccharide breakdown of cell walls in cucumber roots grown with calcium starvation. Plant Physiol. 76:633-637.
10. Liao, C.-H. 1989. Analysis of pectate lyases produced by soft rot bacteria associated with spoilage of vegetables. Appl. Environ. Microbiol. 55:1677-1683.
11. Liao, C.-H. 1991. Cloning of pectate lyase gene *pel* from *Pseudomonas fluorescens* and detection of sequences homologous to *pel* in *Pseudomonas viridiflava* and *Pseudomonas putida.* J. Bacteriol. 173:4386-4393.
12. Liao, C.-H., Hung, H.-Y., and Chatterjee, A. K. 1988. An extracellular pectate lyase is the pathogenicity factor of the soft-rotting bacterium *Pseudomonas viridiflava.* Mol. Plant-Microbe Interact. 1:199-206.
13. Liao, C.-H., McCallus, D. E., and Gaffney, T. D. 1992. Genetic analysis of the *rep* locus that regulates extracellular enzymes and exopolysaccharide production in *Pseudomonas viridiflava.* (Abstr.) Page 184 in: Proc. Int. Symp. Mol. Plant-Microbe Interact., 6th.
14. Liao, C.-H., Sasaki, K., Nagahashi, G., and Hicks, K. B. 1992. Cloning and characterization of a pectate lyase gene from the soft-rotting bacterium *Pseudomonas viridiflava.* Mol. Plant-Microbe Interact. 5:301-308.
15. Liao, C.-H., and Wells, J. M. 1987. Diversity of pectolytic, fluorescent pseudomonads causing soft rots of fresh vegetables at produce markets. Phytopathology 77:673-677.
16. McCallus, D. E., and Liao, C.-H. 1992. Biochemical and genetic analysis of proteases produced by the soft-rotting bacterium *Pseudomonas fluorescens.* (Abstr.) Page 189 in: Proc. Int. Symp. Mol. Plant-Microbe Interact. 6th.
17. McGuire, R. G., and Kelman, A. 1984. Reduced severity of Erwinia soft rot in potato tubers with increased calcium content. Phytopathology 74:1250-1256.
18. McGuire, R. G., and Kelman, A. 1986. Calcium in potato tuber cell walls in relation to tissue maceration by *Erwinia carotovora* pv. *atroseptica.* Phytopathology 76:401-406.
19. McKellar, R. C., and Cholette, H. 1986. Possible role of calcium in the formation of active extracellular proteinase by *Pseudomonas fluorescens.* J. Appl. Bacteriol. 60:37-44.
20. McQueen-Ross, D. A., and Schottel, J. L. 1987. Purification and characterization of a novel extracellular esterase from pathogenic *Streptomyces scabies* that is inducible by zinc. J. Bacteriol. 169:1967-1971.
21. Nasuno, S., and Starr, M. P. 1966. Pectic enzymes of *Pseudomonas marginalis.* Phytopathology 56:1414-1415.
22. Pagel, W., and Heitefuss, R. 1989. Calcium content and cell wall polygalacturonans in potato tubers of cultivars with different susceptibilities to *Erwinia carotovora* subsp. *atroseptica.* Physiol. Mol. Plant Pathol. 35:11-21.
23. Pagel, W., and Heitefuss, R. 1990. Enzyme activities in soft rot pathogenesis of potato tubers: Effect of calcium, pH, and degree of pectin esterification on the activities of polygalacturonase and pectate lyase. Physiol. Mol. Plant Pathol. 37:9-25.
24. Powell, D. A., Morris, E. R., Gidley, M. J., and Rees, D. A. 1982. Conformation and interactions of pectins. II. Influence of residue sequence on chain association in calcium pectate gel. J. Mol. Biol. 155:517-531.
25. Reverchon, S., Van Gijsegem, F., Rouve, M., Kotoujansky, A., and Robert-Baudouy, J. 1986. Organization of a pectate lyase gene family in *Erwinia chrysanthemi.* Gene 49:215-224.
26. Sasaki, K., and Nagahashi, G. 1989. Autolysis-like release of pectic polysaccharides from regions of cell walls other than the middle lamella. Plant Cell Physiol. 30:1159-1169.
27. Sasaki, K., Nagahashi, G., and Liao, C.-H. 1989. Organization of pectins in potato tuber cell walls and their hydrolysis by pectate lyase from *Pseudomonas viridiflava.* (Abstr.) Phytopathology 79:1199.
28. Schlemmer, A. F., Ware, C. F., and Keen, N. T. 1987. Purification and characterization of a pectin lyase produced by *Pseudomonas fluorescens* W51. J. Bacteriol. 169:4493-4498.
29. Sone, H., Sugiura, J., Itoh, Y., Izaki, K., and Takahashi, H. 1988. Production and properties of pectin lyase in *Pseudomonas marginalis.* Agric. Biol. Chem. 52:3205-3207.
30. Stäb, M. R., and Ebel, J. 1987. Effects of Ca^{2+} on phytoalexin induction by fungal elicitor in soybean cells. Arch. Biochem. Biophys. 257:416-423.
31. Tzeng, K.-C., McGuire, R. G., and Kelman, A. 1990. Resistance of tubers from different potato cultivars to soft rot caused by *Erwinia carotovora* subsp. *atroseptica.* Am. Potato J. 67:287-305.
32. Volpin, H., and Elad, Y. 1991. Influence of calcium nutrition on susceptibility of rose flowers to Botrytis blight. Phytopathology

81:1390-1394.
33. Zucker, M., and Hankin, L. 1970. Regulation of pectate lyase synthesis in *Pseudomonas fluorescens* and *Erwinia carotovora*. J. Bacteriol. 104:13-18.
34. Zucker, M., and Hankin, L. 1970. Effectiveness of ethylene diamine-tetraacetic acid (EDTA) in controlling soft rot of potato. Plant Dis. Rep. 54:863-865.
35. Zucker, M., Hankin, L., and Sands, D. 1972. Factors governing pectate lyase synthesis in soft rot and non-soft rot bacteria. Physiol. Mol. Plant Pathol. 2:59-67.