

Elevation and Release of Cell-Associated Pectate Lyase in *Erwinia chrysanthemi* by Lithium and Sodium Chloride

John P. Mildenhall, William A. Lindner, Bernard A. Prior, and Kim Tutt

First and fourth authors: Department of Genetics and Plant Protection, University of Fort Hare, Alice, Ciskei, South Africa. Second author: Department of Biochemistry, University of Fort Hare. Third author: Department of Microbiology, University of the Orange Free State, Bloemfontein, South Africa.

Research project no. AGR73004, University of Fort Hare.

The authors acknowledge financial support from the Council for Scientific and Industrial Research, Pretoria; the Central Research Fund, University of the Orange Free State; and the Research Committee, University of Fort Hare.

The technical assistance of Florence le Maire is appreciated.

Accepted for publication 10 August 1987.

ABSTRACT

Mildenhall, J. P., Lindner, W. A., Prior, B. A., and Tutt, K. 1988. Elevation and release of cell-associated pectate lyase in *Erwinia chrysanthemi* by lithium and sodium chloride. *Phytopathology* 78:213-217.

When *Erwinia chrysanthemi* was grown at a water activity (a_w) value of 0.990 in the presence of LiCl (1.4% w/v) or NaCl (1.4% w/v), the cell-associated levels of pectate lyase (Pel) were 10- to 20-fold greater than in cells grown in the medium (0.998 a_w) without these salts. Upon addition of these salts to the growth medium during the mid-exponential phase of growth, secretion of extracellular Pel ceased for 60 min and thereafter recommenced. Cell-associated levels of Pel increased rapidly during this period. Cells with elevated levels of Pel released the enzyme when

resuspended in buffer or buffer plus sucrose (0.990 a_w); however, little or no Pel was released when the cells were resuspended in buffer plus LiCl, NaCl, KCl, or sorbitol at 0.990 a_w . Release of Pel was accompanied by the release of acid phosphatase (ACP). Pel release was temperature-dependent over the range investigated (15–30 C). Chloroform treatment of cells released Pel and ACP but not β -galactosidase, which indicates that cell-associated Pel is accumulated in the periplasm.

Additional key words: outer membrane, periplasmic enzyme, polygalacturonic acid transeliminase, protein export.

In vitro studies over the past 8 yr on the water relations of *Erwinia chrysanthemi* pv. *zeae* have shown that the specific growth rate of this organism decreases rapidly with the lowering of the water activity of the growth medium to an a_w value of 0.97, below which no growth occurs (9). The production of pectate lyase (Pel; Enzyme Commission [EC] 4.2.2.2) under these conditions was affected mainly by the type of solute used to adjust the a_w of the growth medium rather than the a_w per se (10). Two solutes, lithium chloride (LiCl) and sodium chloride (NaCl), at 0.990 a_w (1.4% w/v) elevated the levels of cell-associated Pel approximately 17-fold. This effect was obtained either by growing *E. chrysanthemi* in the medium containing the appropriate salt concentration or by adding the salt solution to the culture at the mid-exponential phase of growth following initial growth in double-strength basal medium (10).

Several patterns of Pel synthesis and excretion have been reported in *Erwinia*, *Yersinia*, and *Klebsiella* by Chatterjee et al (3). Pel activity in their strain of *E. chrysanthemi* was located mainly in the extracellular fluid and very low levels were cell-associated. A similar Pel distribution was found in our studies when *E. chrysanthemi* was grown in a dilute medium (10). The altered pattern of Pel production observed in cells grown in the presence of LiCl and NaCl prompted us to investigate this effect in more detail. We studied the temporal response of Pel elevation after the addition of LiCl and NaCl, the influence of metabolic and protein synthesis inhibitors on the LiCl and NaCl effect, and the role of temperature and composition of the resuspension medium on the release of Pel from washed cells. The results show that in the presence of LiCl and NaCl, the cell-associated Pel accumulates in the periplasm. Pel accumulation depends on protein synthesis, and its subsequent release from washed cells depends on the nature of the solute in the resuspension fluid. The significance of these findings in relation to the ecology of *E. chrysanthemi* is discussed.

MATERIALS AND METHODS

Organism, medium, and inoculum. A local isolate of *E. chrysanthemi* from maize, designated as strain FH-1 (5), was used in all experiments. The yeast extract salts (YS) medium contained (grams per liter): NH_4NO_3 , 0.40; Na_2HPO_4 , 1.42; KH_2PO_4 , 0.27; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.24; and yeast extract, 0.30. In experiments where Pel and ACP were studied, sodium polypectate (NaPP, 1.80 g/L) was used as carbon source in the YS medium. YS medium containing glycerol (4 g/L) and isopropyl- β -D-thiogalactoside (0.5 mM) as inducer was used for studies on the production of β -galactosidase. The NaPP was purchased from Raltech Inc., Madison, WI, and yeast extract was purchased from Difco Laboratories, Detroit, MI. All of the other chemicals were obtained from Merck, Darmstadt, West Germany. One milliliter of inoculum from an overnight culture was used to seed flasks for all experiments.

Addition of LiCl and NaCl during mid-exponential phase of growth. The cells were grown on an orbital shaker (180 rpm, 30 C) in 250-ml Erlenmeyer flasks, each equipped with side-arm cuvettes and containing 20 ml of double-strength NaPP-YS medium. When the cultures had reached an A_{600} of 0.30 (approximately 1.8×10^8 cells per milliliter), the flask contents were pooled aseptically and redistributed to the flasks (20 ml/flask), thereby ensuring a uniform bacterial suspension in all flasks. One 20-ml sample was retained for the determination of cell-associated and extracellular Pel. The control flasks each received 20 ml of sterile distilled water, whereas the experimental flasks each received 20 ml of sterile NaCl (or LiCl) solution to give a final concentration of 14.14 g NaCl/1,000 g medium (or 14.00 g LiCl), which is equal to an a_w value of 0.990 as determined psychrometrically (11). In another series of experiments, chloramphenicol was added to the culture at a final concentration of 150 $\mu\text{g}/\text{ml}$ 15 min before water or NaCl was added.

The cultures from the control and experimental flasks were harvested at 0, 20, 40, 60, and 80 min after treatment by transferring the contents of each flask to a 50-ml centrifuge tube

embedded in an ice/salt mixture to ensure rapid cooling. Thereafter, the tubes were centrifuged ($6,000 \times g$ for 20 min at 4 C) and the supernatant fluid was assayed for extracellular Pel activity.

Cell-associated Pel activity of the pelleted cells was determined after washing once in 10 ml of YS medium (control) or medium containing LiCl (or NaCl) in the case of cells grown in LiCl (or NaCl). The suspensions were centrifuged as above and the supernatant fluids were discarded. The pellets were resuspended in 2-ml phosphate-saline buffer (Na_2HPO_4 , 0.01 M; K_2HPO_4 , 0.01 M; NaCl, 14 g/L; pH 7.2), sonicated, and centrifuged as described previously (10). The supernatant fluid was assayed for cell-associated Pel as described below.

Release of cell-associated Pel. One hour after water or NaCl solution was added to cultures of *E. chrysanthemi*, flask contents were centrifuged ($12,000 \times g$ for 10 min at 4 C) and the pellets were resuspended in 5 ml of one of the following solutions containing 150 $\mu\text{g}/\text{ml}$ chloramphenicol to inhibit protein synthesis: growth medium only or media with NaCl, KCl, sucrose, or sorbitol, each at 0.990 a_w . These suspensions were harvested immediately (0 min) and after 30-min incubation at 30 C. After centrifugation, the supernatant fluid was assayed for released Pel. The pellets were resuspended in phosphate-saline buffer, sonicated, and centrifuged as described above. The supernatant fluid was assayed for cell-associated Pel and protein (7).

Simultaneous release of Pel and ACP (EC 3.1.3.2). Cultures were grown as described above, except that experimental flasks received LiCl at the mid-exponential phase of growth. The pellets from the control flasks were washed by resuspension in 10 ml of Tris (hydroxymethyl) aminomethane-HCl buffer (0.01 M, pH 7.2) containing chloramphenicol (THC buffer) and resuspended in 2 ml of the same buffer. Five of these suspensions were pooled (10 ml), and this volume was divided into one 2-ml and two 4-ml samples. The 2-ml suspension was sonicated to determine cell-associated Pel. The 4-ml samples were centrifuged ($6,000 \times g$ for 20 min at 4 C) and the supernatant fluids were discarded. The one pellet was resuspended in 4 ml of THC buffer, the other in 4 ml of THC buffer plus LiCl. The cells grown in LiCl were washed in THC buffer plus LiCl (14 g/L buffer) and subsequently treated the same as the controls. The washed cell suspensions were incubated at 30 C on a reciprocal metabolic shaker, 1-ml aliquots were removed at 0, 20, 40, and 60 min and centrifuged in a microfuge ($12,000 \times g$ for 2 min at 4 C), and the supernatant fluid was assayed for ACP and Pel. The above series of experiments was repeated using NaCl in place of LiCl. In addition, release of Pel and ACP from washed cell suspensions was followed over a 60-min incubation period at 15, 20, 25, and 30 C.

The effect of various inhibitors on the release of Pel and ACP from cell suspensions incubated at 30 C was studied by adding either glucose (a catabolite repressor of Pel), fluoroacetate, or potassium cyanide to both the washing and resuspension buffer at a final concentration of 10, 1, and 1 mM, respectively. Chloramphenicol was omitted from the buffer in these experiments.

Release of periplasmic enzymes. The method of Ames et al (1) was used to release periplasmic proteins. The cells received NaCl in the mid-exponential growth phase and were washed in the growth medium containing chloramphenicol. All subsequent steps were done in the presence of chloramphenicol and NaCl. The periplasmic fluid was assayed for Pel, ACP, and β -galactosidase.

Enzyme assays. Pel activity was assayed by mixing a 100- μl enzyme sample with 2.9 ml substrate (0.25% w/v NaPP in 0.05 M Tris-HCl buffer, pH 8.5, containing 37.5 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), and measuring the increase in absorbance at 230 nm on a Beckman model 35 spectrophotometer. Enzyme activity was calculated as described previously (9). ACP was assayed by adding a 100- μl enzyme sample to 1 ml substrate solution (1.65 mg disodium *p*-nitrophenylphosphate from Sigma Chemical Company, St. Louis, MO, per milliliter in 0.05 M sodium acetate buffer, pH 4.5, containing 6.8 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per 100 ml buffer) in a water bath at 30 C. After 20 min incubation, 4 ml of 0.1 M NaOH was added to stop the reaction. A blank determination for each sample received enzyme solution after the addition of NaOH. Enzyme activity was

calculated from the sample A_{400} after subtracting the blank value and using a molar extinction coefficient for *p*-nitrophenol of 22,000 absorbance units per mole. β -galactosidase (EC 3.2.1.23) activity was assayed as described previously (5) in whole cells and in the supernatants obtained after chloroform treatment.

RESULTS

Effect of LiCl and NaCl addition during the mid-exponential phase of growth. Extracellular Pel production increased consistently after the addition of water (control flasks 0.998 a_w) to cells in the mid-exponential phase of growth (Fig. 1A). In contrast, the level of extracellular Pel remained constant for approximately 60 min after the addition of NaCl and thereafter increased (Fig. 1B). This lag in extracellular Pel secretion corresponds with the growth lag that follows solute addition (10). Cell-associated Pel remained constant at a low level in the control cells, whereas an increase occurred over an 80-min period in cells receiving NaCl (Fig. 1). This elevation was observed within 20 min of NaCl addition during the lag period of extracellular Pel production. A similar pattern was observed when LiCl replaced NaCl.

Low levels of Pel were observed in cells where chloramphenicol was added before NaCl (0.16 units per milligram of protein) or water (0.12 units per milligram of protein); however, cells receiving NaCl in the absence of chloramphenicol yielded 0.70 units Pel per milligram of protein. These results indicate that elevation of cell-associated Pel depends on *de novo* protein synthesis.

Release of cell-associated Pel from *E. chrysanthemi*. Cells of *E. chrysanthemi* grown in the presence of NaCl (0.990 a_w) released 0.24 units of cell-associated Pel when resuspended in either the basal medium or the basal medium plus sucrose (0.990 a_w ; Table 1). In contrast, only 0.03, 0.01, and 0.02 units of cell-associated Pel were released into the medium containing LiCl, NaCl, and KCl, respectively (Table 1). However, 0.09 units were released into the medium containing sorbitol.

Release of Pel and ACP. Both Pel and ACP were released from LiCl-grown cells resuspended in THC buffer (Fig. 2A and B). Control cells, resuspended in THC buffer and LiCl, released ACP only (Fig. 2B). A slight release of Pel was observed from cells receiving water in mid-exponential growth phase and subsequently resuspended in buffer (Fig. 2A), whereas no Pel release occurred from such cells resuspended in buffer plus LiCl. The release of

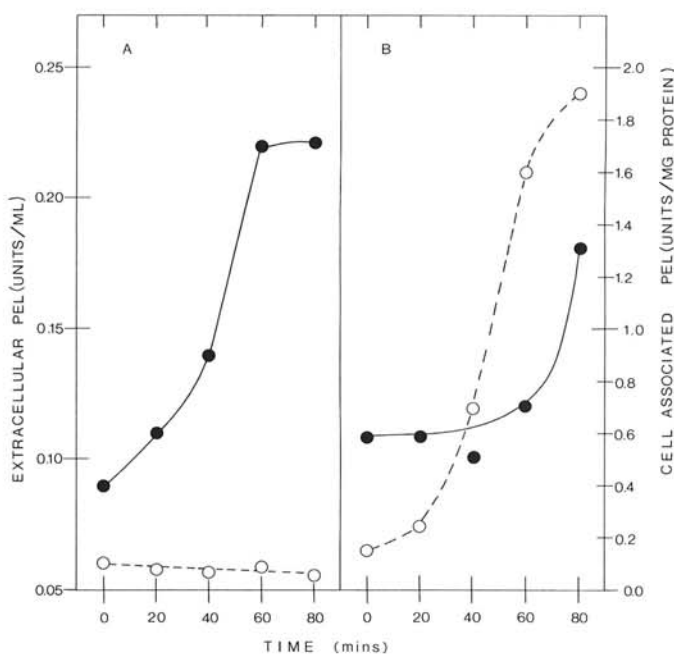


Fig. 1. Production of extracellular (●-●) and cell-associated (○-○) pectate lyase (PEL) by *Erwinia chrysanthemi* upon addition of A, water (0.998 a_w) or B, NaCl (0.990 a_w) to a culture in the mid-exponential phase of growth.

cell-associated Pel and ACP from cell suspensions was not affected by glucose, fluoroacetate, and potassium cyanide.

Effect of temperature on the release of cell-associated Pel and ACP. Very little Pel or ACP was released from cell suspensions incubated at 15 C (Fig. 3A and B). However, the release of both

enzymes from cells grown in the medium plus NaCl and resuspended in THC buffer increased between 15 and 30 C. Cells receiving water in the mid-exponential growth phase and subsequently resuspended in THC buffer plus NaCl released comparatively large amounts of ACP, especially at 30 C (36 units per milliliter). A similar release of Pel was not observed from cells treated in this way, because they had very low levels of cell-associated Pel.

Location of cell-associated Pel. When cells receiving NaCl at the mid-exponential phase of growth were treated with chloroform, 86 ± 3 , 47 ± 4 , and $1.6 \pm 0.3\%$ of the total cell-associated Pel, ACP, and β -galactosidase, respectively, were released.

TABLE I. Release of pectate lyase (Pel) from cells of *Erwinia chrysanthemi* grown at 0.990 a_w (NaCl) and resuspended at 30 C in basal medium (control) or basal medium plus solute (0.990 a_w)

Solute	Units Pel released over 30 min
Basal medium only	0.24 ± 0.07^a
NaCl	0.01 ± 0.01
LiCl	0.03 ± 0.01
KCl	0.02 ± 0.01
Sorbitol	0.09 ± 0.02
Sucrose	0.24 ± 0.04

^a Each value represents the mean of three experiments \pm standard error.

DISCUSSION

The data reported in this paper show that certain solutes govern the elevation and release of cell-associated Pel in *E. chrysanthemi*. The elevation of cell-associated Pel levels occurred simultaneously with the temporary cessation of extracellular Pel production, thereby indicating that LiCl and NaCl interrupt secretion of

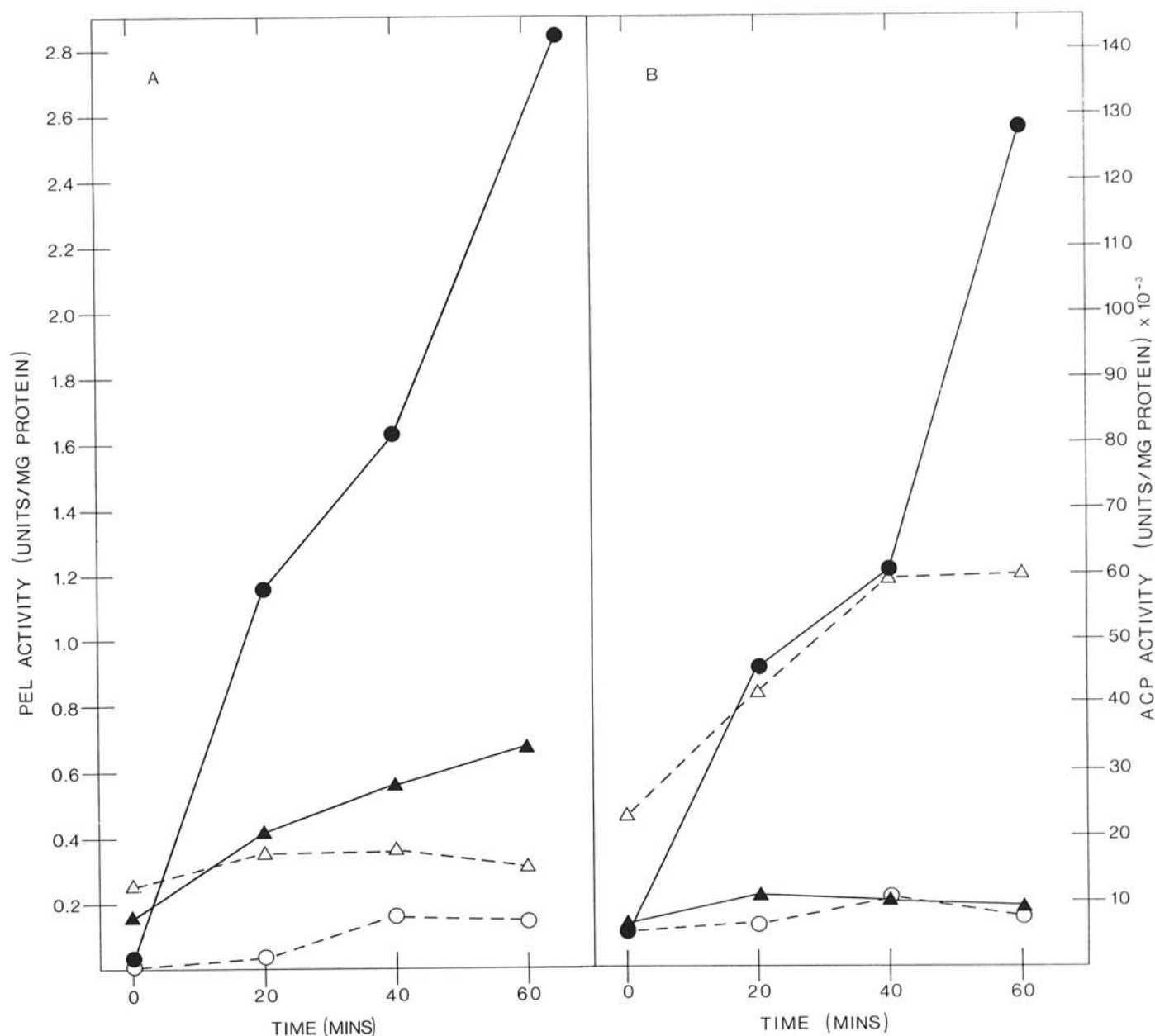


Fig. 2. Release of **A**, pectate lyase (PEL) and **B**, acid phosphatase (ACP) from washed cells of *Erwinia chrysanthemi* resuspended at 30 C in the presence (dashed line) or absence (solid line) of LiCl, as described in the text. Cells were obtained by incubating *E. chrysanthemi* for 1 hr after the addition of water (0.998 a_w) (△, ▲) or LiCl (0.990 a_w) (○, ●) to a culture in the mid-exponential phase of growth.

extracellular enzyme. Interrupted enzyme secretion causes Pel to accumulate in association with the cell until a point is reached where normal Pel export resumes. Neither ACP nor β -galactosidase levels were affected by the addition of LiCl and NaCl to the culture in the mid-exponential phase of growth (*unpublished data*). These enzymes are found in the periplasm and cytoplasm, respectively, and are not normally secreted into the growth medium. In contrast, Pel is mainly an extracellular enzyme (15) and these salts apparently exerted their effect on Pel export.

The production of extracellular Pel by *E. chrysanthemi* ostensibly involves two distinct steps (2, 12): initial translocation of the enzyme from the cytoplasm to the periplasm, followed by a subsequent secretion from the periplasm to the external medium. The latter step is regulated by the *out* gene (4,15). Our results indicate that the addition of LiCl or NaCl to the growth medium temporarily inhibits one of the above-mentioned steps.

The release of Pel from cells in the presence of chloramphenicol suggests that the enzyme was not released from the cytoplasm, because translocation of protein across the cytoplasmic membrane involves protein synthesis (13). The fact that release was not subject to catabolite repression by glucose further supports this conclusion.

In contrast, two lines of evidence suggest that, in the presence of LiCl and NaCl, Pel accumulates in the periplasm. Firstly, in resuspension experiments, both ACP and Pel were released simultaneously over a range of temperatures, suggesting a common place of origin. Secondly, both enzymes were released following chloroform treatment. Furthermore, chloroform treatment failed to release significant amounts of intracellular β -galactosidase, indicating that only periplasmic components were liberated. Therefore, LiCl and NaCl exert their effect primarily on

the movement of Pel across the outer membrane. Although little is known about protein export across the outer membrane, our results do indicate that release of Pel from the periplasm is passive, because neither fluoroacetate nor KCN inhibited the process. The inhibition of Pel release by LiCl and NaCl but not sucrose shows that the process does not depend on the maintenance of isotonic conditions. The partial inhibition of Pel release by sorbitol indicates that the mechanism is not solely dependent on an ionic effect.

Although pectic enzymes have been extensively studied in *Erwinia* spp. (4), the solute effects reported in this paper have received little attention (10), and we are not aware of similar effects that have been described for any other extracellular enzyme produced by bacteria. The ability of *E. chrysanthemi* to accumulate cell-associated Pel in response to certain environmental changes and subsequently to release the enzyme under suitable conditions may be significant in relation to the infection process. Moreover, the enhanced release of cell-associated Pel with increasing temperature may be related to the finding that temperatures of 30 C and higher are required for optimal infection of plants (6).

Little is known about the physiological changes that phytopathogenic bacteria undergo in the absence of living host tissue (8,14), and mechanisms that enable them to survive and reinfect new tissue must exist. One such mechanism could be the elevation of cell-associated Pel, an effect that could occur when *E. chrysanthemi* is subjected to desiccation (solute effects) in infected crop residues. Upon subsequent exposure to free water on new host tissues, the Pel would be released, thereby enhancing the infection process.

The response of *E. chrysanthemi* to LiCl and NaCl may be used

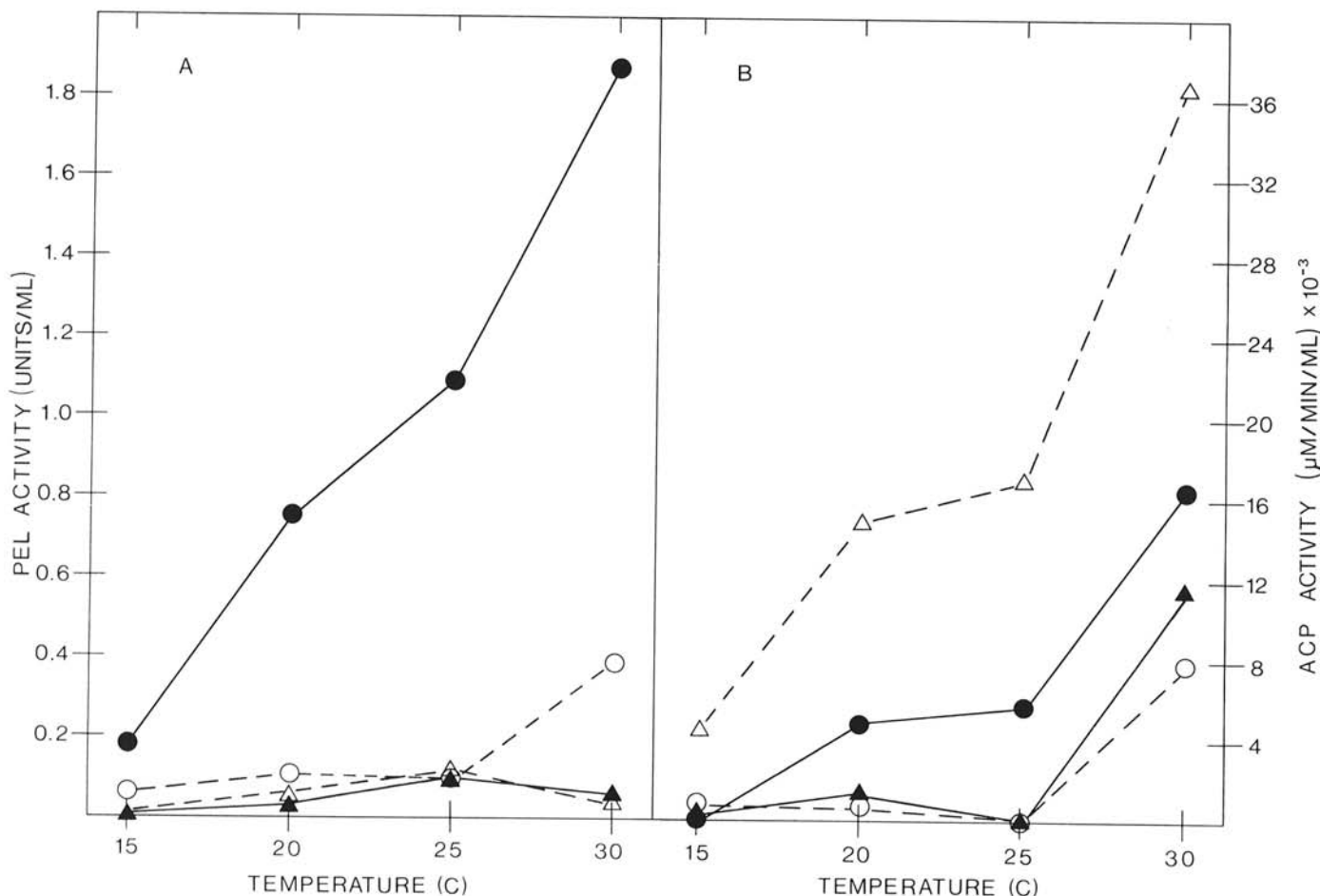


Fig. 3. Release of A, pectate lyase (PEL) and B, acid phosphatase (ACP) over 60 min from washed cells of *Erwinia chrysanthemi* resuspended at different temperatures in the presence (dashed line) or absence (solid line) of NaCl, as described in the text. Cells were obtained by incubating *E. chrysanthemi* for 1 hr after the addition of water (0.998 a_w) (Δ, ▲) or NaCl (0.990 a_w) (○, ●) to a culture in the mid-exponential phase of growth.

as a taxonomic criterion or to study the expression of cloned *pel* genes. The manipulation of periplasmic Pel levels using LiCl and NaCl may also be a useful tool to investigate the mechanism of Pel export across the outer membrane and to study the role of periplasmic Pel accumulation in pathogenesis.

LITERATURE CITED

1. Ames, G. F-L., Prody, C., and Kustu, S. 1984. Simple, rapid and quantitative release of periplasmic proteins by chloroform. *J. Bacteriol.* 160:1181-1183.
2. Andro, T., Chambost, J.-P., Kotoujansky, A., Cattaneo, J., Bertheau, Y., Barras, F., Van Gijsegem, F., and Coleno, A. 1984. Mutants of *Erwinia chrysanthemi* defective in secretion of pectinase and cellulase. *J. Bacteriol.* 160:1199-1203.
3. Chatterjee, A. K., Buchanan, G. E., Behrens, M. K., and Starr, M. P. 1979. Synthesis and excretion of polygalacturonic acid transeliminase in *Erwinia*, *Yersinia* and *Klebsiella* species. *Can. J. Microbiol.* 25:94-102.
4. Collmer, A., and Keen, N. T. 1986. The role of pectic enzymes in plant pathogenesis. *Annu. Rev. Phytopathol.* 24:383-409.
5. Gray, J. S. S., Lindner, W. A., Brand, J. M., and Mildenhall, J. P. 1986. Lactose and melibiose metabolism in *Erwinia chrysanthemi*. *J. Bacteriol.* 168:886-891.
6. Hartman, J. R., and Kelman, A. 1973. An improved method for the inoculation of corn with *Erwinia* spp. *Phytopathology* 63:658-663.
7. Hartree, E. F. 1972. Determination of protein: A modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* 48:422-427.
8. Leben, C. 1981. How plant-pathogenic bacteria survive. *Plant Dis.* 65:633-637.
9. Mildenhall, J. P., Prior, B. A., and Trollope, L. A. 1981. Water relations of *Erwinia chrysanthemi*: Growth and extracellular pectic acid lyase production. *J. Gen. Microbiol.* 127:27-34.
10. Mildenhall, J. P., and Prior, B. A. 1983. Water relations of *Erwinia chrysanthemi*: Intracellular and extracellular pectate lyase production. *J. Gen. Microbiol.* 129:3019-3025.
11. Prior, B. A., Casaleggio, C., and van Vuuren, H. J. J. 1977. Psychrometric determination of water activity in the high a_w range. *J. Food Protect.* 40:537-539.
12. Pugsley, A. P., and Schwartz, M. 1985. Export and secretion of proteins by bacteria. *FEMS Microbiol. Rev.* 32:3-38.
13. Randall, L. L., and Hardy, S. J. S. 1984. Export of protein in bacteria. *Microbiol. Rev.* 48:290-298.
14. Schuster, M. L., and Coyne, D. P. 1974. Survival mechanisms of phytopathogenic bacteria. *Annu. Rev. Phytopathol.* 12:199-121.
15. Thurn, K. K., and Chatterjee, A. K. 1985. Single-site chromosomal Tn 5 insertions affect the export of pectolytic and cellulolytic enzymes in *Erwinia chrysanthemi*: EC 16. *Appl. Environ. Microbiol.* 50:894-898.