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Effect of Sodium Chloride and Glycine Betaine on Growth and Pectate Lyase Production by *Erwinia carotovora* subsp. *carotovora*

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Abstract: *E. carotovora* subsp. *carotovora* was grown in YS medium containing different NaCl concentrations. The growth was stimulated, reduced and totally inhibited in presence of 1.5, 3 and 6% NaCl, respectively. Addition of glycine betaine increased the growth in presence of NaCl, but not in its absence, in comparison with the control. Assays of extracellular and cell associated pectate lyase produced by *E. carotovora* in NaPP-YS medium containing 1.5 and 3% NaCl revealed that the extracellular PL activity was inhibited in presence of NaCl relative to the control. On the other hand, the level of the cell associated enzyme was increased with increasing NaCl concentration. When addition of glycine betaine to the growth medium, the activity of the extracellular PL was increased even in the control medium devoid of NaCl. Also, glycine betaine released the intracellular PL, this was evidenced by the reduction of the cell associated PL activity in its presence compared with the control (absence of glycine betaine).

Key words: *Erwinia carotovora*, glycine betaine, sodium chloride

INTRODUCTION

When bacterial cells are exposed to high external osmolarity they respond by accumulating a number of solutes which are termed osmoprotectants and alleviate osmotic inhibition[1-3]. Glycine betaine (N,N,N-trimethylglycine) is an important compound of these osmoprotectants. Few photoautotrophic bacterial species can carry out the complete synthesis of glycine betaine[4-6]. Whereas most other bacteria are unable to do so and therefore they are dependant on the transport of this compound, or its precursor, cholin[7-9] for its accumulation. Subsequently, the ability of glycine betaine to stimulate the respiration rate of a halophilic bacterium in media of elevated NaCl concentration was observed by Rafaeli-Eshkol and Avi-Dor[10]. It has been reported that this compound is a potent osmoprolectant for members of the family *Enterobacteriaceae*[11].

Several studies investigated the role of osmoprotectants in growth and PL production by *E. chrysanthemi* under osmotic stress[12-16], whereas few studies were carried out on *E. carotovora* subsp. *carotovora* in this respect[17], although it has a wide host range and it is also considered as a destructive phytopathogen.

This study was undertaken to investigate the role of glycine betaine on growth and PL production by *E. carotovora* subsp. *carotovora* in presence and absence of NaCl.

MATERIALS AND METHODS

Bacterial strain: *Erwinia carotovora* subsp. *carotovora* pep 2A which was isolated from rotted pepper fruits and produces pectate lyase[18] is used in this study. This isolate is maintained on nutrient agar slopes, stored at 4°C and subcultured monthly.

Medium: This isolate was grown in sodium polypectate yeast extract salt medium (NaPP-YS) that described by Mildenhall *et al.*[19]. It contained (grams per liter): NH_4NO_3, 0.40; Na_2HPO_4, 1.42; KH_2PO_4, 0.27; $MgSO_4.7H_2O$, 0.24 and yeast extract, 0.30. In experiments where pectate lyase activity was studied, sodium polypectate, 1.80 g L^{-1} was used. The medium was adjusted to pH 7.0 and dispensed into 20 mL aliquots in 250 mL flaks and sterilized, then flasks were inoculated each with 1 mL overnight culture and incubated at 30°C by shaking. When the cultures had reached on OD_{600} of 0.3 (approximately $1.8x10^8$ cells mL^{-1}), the flask contents were pooled aseptically and redistributed (20 mL/flask). The control flasks each received 20 mL of sterile distilled water, whereas the experimental flasks each received 20 mL of sterile NaCl solutions to yield a final concentration of 1.5, 3 and 6%.

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The remaining flasks received the same NaCl solution supplemented with glycine betaine (0.5 mol L^{-1} stock solution, filter sterilized and 0.1 mL was added to each flask). The growth was estimated and pectate lyase activity was assayed in the control and experimental flasks at 0, 20, 40, 60, 80, 100 and 120 min. The growth was estimated by measuring OD_{600}.

Preparation of pectate lyase solution and enzyme assay: The contents of each flask was centrifuged (6.000 g for 20 min at 4°C) and the supernatant was assayed for extracellular pectate lyase (PL) activity by the thiobarbituric acid method[20]. Cell associated pectate lyase activity was determined by washing the pellets in 10 mL YS medium in case of the control or medium containing the related NaCl concentration. The pellets were responded in 2 mL phosphate saline buffer (Na_2HPO_4, 0.01 M; K_2HPO_4, 0.01 M; NaCl 14 g L^{-1}; pH 7.2), sonicated at 100% amplification and 5 pulses of 10 sec with an interruption of 30 sec for cooling in an ice bath and centrifuged. The supernatant fluid was assayed for PL activity.

Determination of protein concentration: This was determined by the method of Bradford[21].

RESULTS AND DISCUSSION

Erwinia carotovora subsp. *carotovora* was grown in YS medium containing 0, 1.5, 3 and 6% NaCl with or without glycine betaine. The obtained results showed that the growth was stimulated in the presence of 1.5% NaCl (Fig.1B), reduced at 3% (Fig.1C) and totally inhibited with 6% NaCl (Fig.1D), relative to the control with no NaCl. In contrast to this result, Gouesbet *et al.*[13] found that *E. chrysanthemi* growth was optimal in the absence of NaCl and was significantly affected by up to 0.3 M NaCl. However, above this value they found that the growth rate was dramatically reduced and at concentrations greater than 0.6 M NaCl the growth was totally inhibited. Also the inhibition of the enterobacterial growth in presence of NaCl has been reported[3,12,19].

In this study, glycine betaine did not affect the bacterial growth in the control medium devoid of NaCl (Fig.1A). It affected the growth only in presence of NaCl (Fig. 1B-D). Similarly, Prior *et al.*[12] found that glycine betaine stimulated the growth of two strains of *E. chrysanthemi* only in the presence of NaCl. Also, the growth of three strains of *E. chrysanthemi* on 0.3 M NaCl medium was improved by the addition of glycine betaine[16]. Moreover, uptake of glycine betaine was observed only in cells grown in medium with high osmolarity[13]. At 1.5% NaCl, the addition of glycine betaine stimulated the growth but an early cessation of the growth was observed (Fig. 1B). In absence of glycine betaine the stationary phase began after 100 min of NaCl addition, whereas in its presence it began after 80 min.

It has been reported that osmoprotectants, including glycine betaine, represented an alternative to the ionic solutes used for osmoregulation by many bacteria at low osmolality and by a few at high osmolality[3]. This is probably because the energy needed to accumulate osmoprotectants is less than that needed for the uptake of K^+ and the synthesis of glutamate[3].

Cayley *et al.*[22] suggested that the beneficial effects of osmoprotectants could be based in part on their ability to increase the water content of the cells. In addition to their role in the recovery and maintenance of osmotic balance, Bourot *et al.*[23] demonstrated that glycine betaine assisted protein folding in a mutant of *E. coli.*

Extracellular and cell associated PL production by *E. carotovora* subsp. *carotovora* in NaPP-YS medium containing 0, 1.5 and 3% NaCl, in absence or presence of glycine betaine was assayed. At 0% NaCl concentration, the maximum level of PL activity was reached after 80 min in absence or presence of glycine betaine, then the enzyme activity was reduced. This reduction could be attributed to the inhibition of enzyme activity by certain metabolites or due to change in pH value. In this treatment, extracellular PL activity was increased in presence of glycine betaine and no change was observed in the activity of the cell associated PL (Fig. 2A).

Although the medium containing 1.5% NaCl stimulated the growth, it showed an inhibitory effect on PL activity (Fig. 2B). Also the extracellular enzyme activity obtained in the medium containing 3% NaCl was inhibited to about 44% of that observed in the control without NaCl (Fig. 2C). On the other hand, the activity of the cell associated enzyme was about 8.33 times as that obtained from the control (Fig. 2C). In contrast to this result, Gouesbet *et al.*[13] found that the increased medium osmolarity resulted first in an induction of PL activity, followed by a shift to the basal level at higher osmolyte concentrations. Furthermore, Hugouvieux-Cotte-Pattat *et al.*[24] found that the *pelE* gene, encoding the major PL in *E. chrysantheni*, was

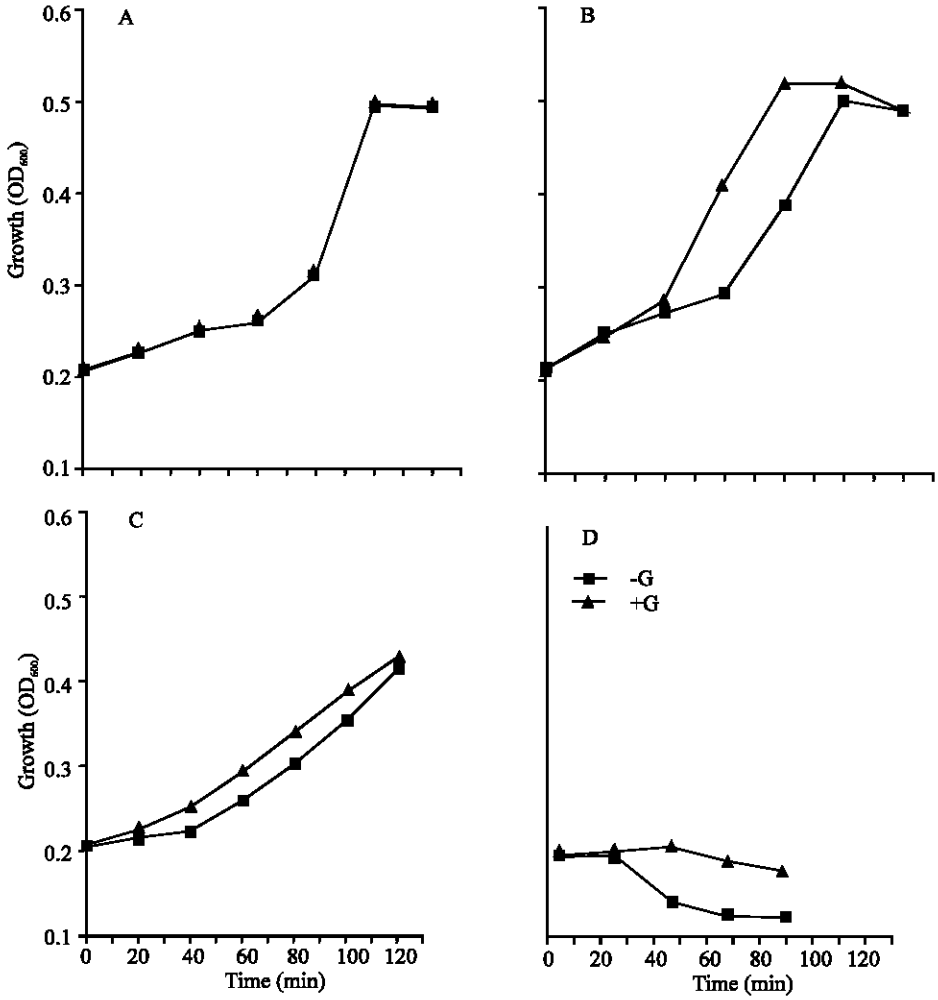

Fig. 1: Growth of *E.c.* Subsp. *carotovora* isolate pep2A in YS medium supplemented with different NaCl concentration in the presence, -▲- and absence -■- of glycine betaine. A: 0% NaCl, B: 1.5% NaCl, C: 3% NaCl, D: 6% NaCl.

strongly induced in the presence of 0.3 M NaCl, while expression of the other *pel* genes was either not affected or weakly repressed by elevated medium osmolarity. However, the inhibition of PL production in presence of NaCl has been reported[12,19].

Addition of glycine betaine to the media containing NaCl increased the extracellular enzyme activity and reduced the activity of the cell associated enzyme. For example, in the medium with 1.5% NaCl the extracellular enzyme activity was increased by 28% after 80 min of NaCl addition and the cell associated PL activity was reduced by 20% after 100 min, relative to the control without glycine betaine.

In an other study, the inhibition of PL excretion by NaCl was coupled to an accumulation of PL in the periplasm[19]. Moreover, Prior *et al.*[12] showed that the periplasmic PL levels in *E. chrysanthemi* were lower in the presence of exogenously added osmoprotectants.

Cayley *et al.*[22] showed that the inhibitory effects of high ionic strength on enzymatic activity are avoided by replacing ions with neutral osmoprotectants. They also found that osmoprotectants stabilize and protect enzymes. This might explain the increased activity of PL when addition of glycine betaine.

It has been reported that plants accumulate osmoprotectants, including glycine betaine, under osmotic stress[25-28]. Based on present results as well as the results obtained by Prior *et al.*[12] it can be suggested that osmoprotectants accumulated in host plants could stimulate growth and PL release by soft rot *Erwinia*.

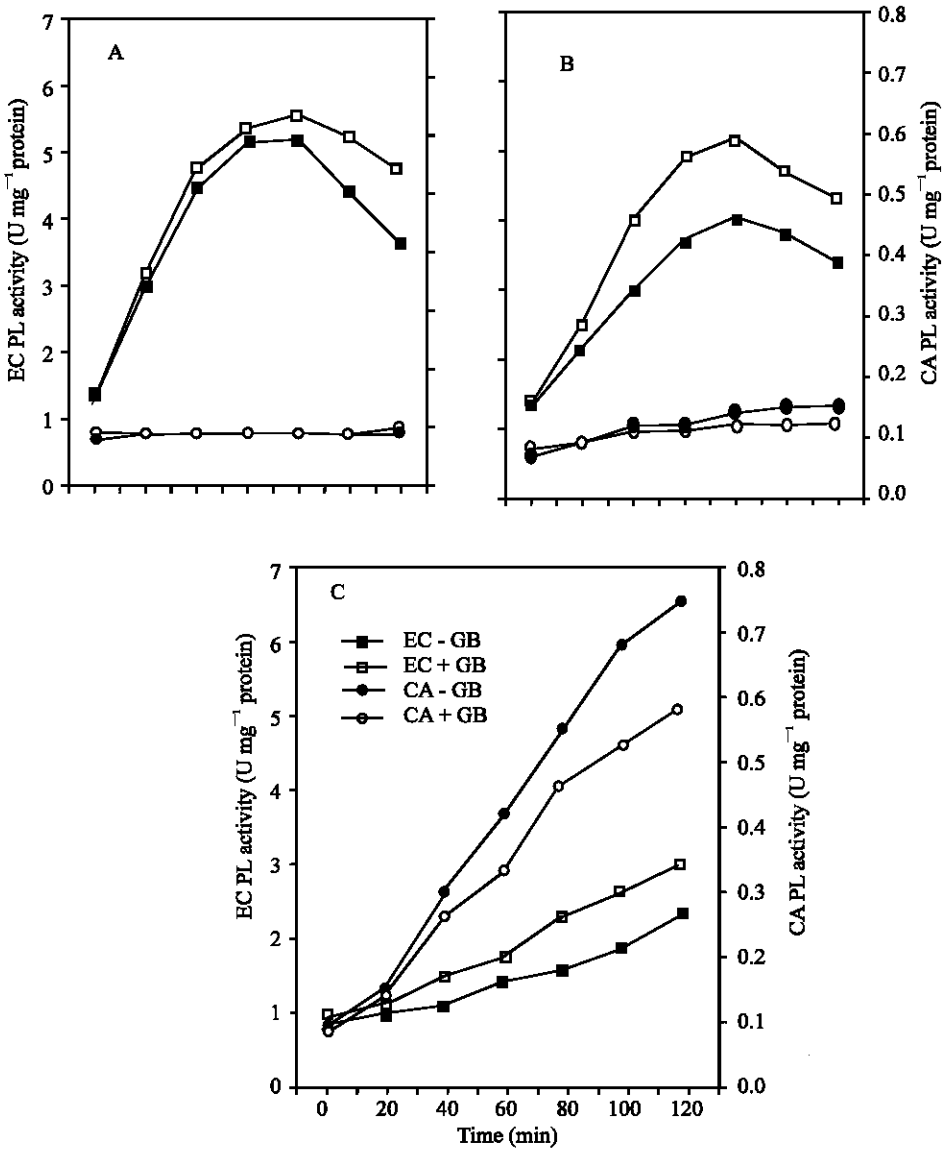

Fig. 2: Production of PL by *E. carotovora* subsp. pep2A in NaPP-YS medium containing NaCl at the concentrations of: A: 0%; B: 1.5% and C: 3% in presence and absence of glycine betaine. -■-, extracellular PL in absence of glycine betaine. -▫-, extracellular PL in presence of glycine betaine. -•-, cell associated PL in absence of glycine betaine. -○-, cell associated PL in presence of glycine betaine

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