***Erwinia chrysanthemi* at high osmolarity: influence of osmoprotectants on growth and pectate lyase production**

Gwenola Gouesbet,¹ Mohamed Jebbar,¹ Sylvie Bonnassie,¹
Nicole Hugouvieux-Cotte-Pattat,² Souad Himdi-Kabbab³ and
Carlos Blanco¹

Author for correspondence: Carlos Blanco. Tel: +33 99 28 61 40. Fax: +33 99 28 67 00.
e-mail: Carlos.Blanco@univ-rennes1.fr

¹ CNRS URA 256,
Département Membranes
et Osmorégulation,
Université de Rennes I,
Campus de Beaulieu,
Avenue du Général
Leclerc, 35042 Rennes
Cedex, France

² CNRS URA 1486-1,
Laboratoire de Génétique
Moléculaire des
Microorganismes, INSA
Bât. 406, 20 Avenue Albert
Einstein, 69621
Villeurbanne Cedex,
France

³ CNRS URA 704, Groupe de
Recherche de
Physicochimie Structurale,
Université de Rennes I,
Campus de Beaulieu,
Avenue de Général Leclerc,
35042 Rennes Cedex,
France

The mechanism of osmotic stress adaptation was investigated in the phytopathogen *Erwinia chrysanthemi*. Growth of the bacterium was inhibited by elevated medium osmolarity, and exogenous glycine betaine, proline, ectoine or pipercolate permitted recovery of growth at inhibitory osmolarity. Osmoprotectants were taken up by transporters induced by elevated osmolarity, and their level of accumulation within the cell was dependent on the osmolarity of the growth medium. The influence of osmolarity and osmoprotectants on the production of pectate lyases (PLs) was investigated. Increased medium osmolarity resulted first in an induction of PL activity, followed by a shift to the basal level at higher osmolyte concentrations. This induction was reversed by osmoprotectants in the medium. The increased PL activity was attributed in part to the induced transcription of the main PL gene, *pelE*, and all the osmoprotectants that were analysed were found to prevent *pelE* induction. PL activity was partially inhibited *in vitro* by high ionic strength but not by elevated concentrations of sugars, and the addition of osmoprotectants at 1 mM had no effect on PL activity *in vitro*.

Keywords: *Erwinia chrysanthemi*, osmoprotectants, pectate lyases, *pel* genes, osmoadaptation

INTRODUCTION

Moisture was recognized by Colhoun (1973) to be an important factor in the infection of plants by pathogenic micro-organisms. A correlation between irrigation and stalk rot of maize suggested that *Erwinia chrysanthemi* is sensitive to desiccation (Hoppe & Kelman, 1969). The phytopathogenic bacterium *E. chrysanthemi* produces extracellular pectate lyases (PLs) which attack components of the plant cell wall (Kotoujansky, 1987). The PL structural genes (*pel*) are organized in five independent operons grouped into two clusters on the bacterial chromosome (Kotoujansky, 1987). The influence of environmental conditions on the total pectinase production and on the expression of the individual *pel* genes was examined in *E. chrysanthemi* 3937, and *pel* transcription proved to be sensitive to many conditions (Hugouvieux-Cotte-Pattat *et al.*, 1992), and their differential expression was observed at

different medium osmolarities. The *pelE* gene, encoding the major pectate lyase, was strongly induced in the presence of 0.3 M NaCl, while expression of the other *pel* genes was either not affected or was weakly repressed by elevated medium osmolarity (Hugouvieux-Cotte-Pattat *et al.*, 1992). Recently, Prior *et al.* (1994) presented opposite results using different strains of *E. chrysanthemi*. These authors showed that secretion of PL in the extracellular medium decreased when water activity was reduced. However, this decrease in extracellular PL was linked to intracellular accumulation of PL. They also demonstrated that growth of *E. chrysanthemi* is limited under osmotic stress and that this effect is reversed in presence of various osmoprotectants (Prior *et al.*, 1994). Nevertheless they did not analyse the behaviour of osmoprotectants. We have previously shown (Talibart *et al.*, 1994) that the mechanism of osmoprotection in *Escherichia coli* (Csonka, 1989; Csonka & Hanson, 1991) is not applicable to all bacteria. Thus, we have analysed the mechanism(s) of osmoregulation in *E. chrysanthemi* strain 3937, especially the behaviour of various osmoprotectants within the cells,

Abbreviation: PL, pectate lyase.

and have demonstrated that variations in PL production result from transcriptional induction of the *pelE* gene.

METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study were *E. chrysanthemi* 3937 (wild-type) and its derivatives A1880 (*pelC::uidA* Km) and A1828 (*pelE::uidA* Km) (Hugouvieux-Cotte-Pattat *et al.*, 1992). Cells were grown on LB medium or in minimal medium M63 (Miller, 1972) at 30 °C. Carbon sources were added at 2 g l⁻¹. Minimal media of elevated osmotic strength were made by adding NaCl, KCl, mannitol, sorbitol or glycerol, and the osmotic pressure was determined by measuring the freezing point with an osmometer. The osmoprotectants glycine betaine, proline, ectoine and pipicolate were generally supplied at a final concentration of 1 mM. Bacterial growth was monitored by OD₅₇₀.

Enzyme assays. Assays of total PL and β -glucuronidase were performed on toluene-permeabilized cultures grown to late exponential-phase to achieve maximal PL production. Total PL activity was determined by the degradation of polygalacturonate to unsaturated products that absorb at 235 nm (Hugouvieux-Cotte-Pattat *et al.*, 1992); specific activity (U) is expressed as μ mol unsaturated products liberated min⁻¹ (mg dry wt)⁻¹. β -Glucuronidase activity was measured by following the degradation of the substrate *p*-nitrophenyl- β -D-glucuronide into *p*-nitrophenol that absorbs at 405 nm (Hugouvieux-Cotte-Pattat *et al.*, 1992); specific activity (U) is expressed as nmol *p*-nitrophenol liberated min⁻¹ (mg dry wt)⁻¹. The results reported are the average of at least three independent experiments, and the SD was less than 10% in each case.

Uptake and behaviour of [¹⁴C]osmoprotectants. [1,2-¹⁴C]glycine betaine was prepared from [1,2-¹⁴C]choline as described by Ikuta *et al.* (1977). [U-¹⁴C]pipicolate was purified from a culture of *Brevibacterium ammoniagenes* ATCC 6872 supplied with [¹⁴C]lysine as described by Gouesbet *et al.* (1992). [¹⁴C]ectoine was biologically prepared from L-[U-¹⁴C]glutamic acid supplied to *Brevibacterium linens* ATCC 9175 as described by Jebbar *et al.* (1992). L-[U-¹⁴C]proline (9.84 GBq mmol⁻¹) was purchased from Amersham-France. Extraction of cellular solutes, paper chromatography, HPLC, electrophoresis and ¹³C NMR analysis of cell extracts were described by Gouesbet *et al.* (1992) and Bernard *et al.* (1993). Uptake assays were carried out as described by Jebbar *et al.* (1992).

Determination of intracellular solute content. The osmoprotectant content of the cells was determined after growth in M63 medium for three generations, with various NaCl concentrations in the presence of 1 mM [¹⁴C]pipicolate (0.9 MBq mmol⁻¹), 1 mM [¹⁴C]glycine betaine (1.3 MBq mmol⁻¹), 1 mM [¹⁴C]proline (1.1 MBq mmol⁻¹) or 1 mM [¹⁴C]ectoine (0.6 MBq mmol⁻¹). Cells were collected by centrifugation (3500 g, 10 min). After ethanolic solute extraction and electrophoretic separation of the soluble fraction (Gouesbet *et al.*, 1992), the radioactivity of each spot was determined by scintillation counting. Twenty percent of the radioactivity was incorporated by the cells during the assay. Results are the mean of at least three independent experiments, and the SD was less than 10%.

RESULTS AND DISCUSSION

Behaviour of osmoprotectants in the cell

The growth rate of *E. chrysanthemi* 3937 was analysed in M63 minimal medium supplemented with various concentrations of NaCl. Growth was optimal in the absence of NaCl and was not significantly affected by up to

Fig. 1. Accumulation of exogenously supplied osmoprotectants in medium of increasing osmolarity. Cells were grown for three generations in M63 medium containing no salt or 0.3, 0.4, 0.5 or 0.7 M NaCl in the presence of: ○, 1 mM [¹⁴C]glycine betaine; □, [¹⁴C]ectoine; ●, [¹⁴C]proline; or ■, [¹⁴C]pipicolate.

0.3 M NaCl. Above this value the growth rate was dramatically reduced, and at concentrations greater than 0.6 M NaCl growth was totally inhibited. As previously described for various *E. chrysanthemi* isolates (Prior *et al.*, 1994), the growth of *E. chrysanthemi* 3937 in medium of inhibitory osmolarity was improved by the presence of glycine betaine, proline or pipicolate. In addition, we observed that ectoine was as effective as glycine betaine in improving the growth of *E. chrysanthemi* 3937. These osmoprotectants were active at a very low concentration (5 μ M).

Uptake of glycine betaine, pipicolate and ectoine was observed only in cells grown in medium of high osmolarity, whereas low-level proline uptake was also observed in cells grown at low osmolarity. Uptake of osmoprotectants was inducible by osmolarity rather than by water activity, since the induction of uptake was observed on addition of NaCl, KCl, mannitol or sucrose, but not on addition of the freely diffusible compound glycerol.

¹³C NMR analysis of ethanolic extracts of cells grown in medium containing 0.5 M NaCl and 1 mM of the various

Fig. 2. Influence of NaCl concentration and osmoprotectants on the production of PL. Cells were grown in M63 medium containing various concentrations of NaCl, in the absence of osmoprotectant (▲), or in the presence of (all 1 mM) glycine betaine (○), proline (●), ectoine (□) or pipecolate (■). Specific activity is expressed as μmol unsaturated products liberated min^{-1} (mg dry wt^{-1}).

osmoprotectants showed that all the osmoprotectants tested were accumulated in the cell and represented the main molecule, since only their specific signals were observed. The level of accumulation of each osmoprotectant was analysed in medium of increasing osmolarity containing 1 mM ^{14}C -labelled osmoprotectant. In each case, the ethanolic soluble fraction containing radioactivity was quantified by scintillation counting (Fig. 1). Since these solutes are not metabolized by the cell, except for proline, the incorporated radioactivity corresponded to the accumulated molecule. The amount of proline determined by HPLC was similar to that deduced from scintillation counting of the proline spot. The intracellular content of each compound increased with medium osmolarity. In cells grown in medium deprived of NaCl (Fig. 1), the content of all molecules was 50–80 nmol (mg dry wt^{-1}). In contrast, this accumulation

increased with medium osmolarity to 1.9, 1.3, 1.2 and 1.5 μmol (mg dry wt^{-1}) for glycine betaine, proline, ectoine and pipecolate, respectively, in medium containing 0.7 M NaCl. For all the NaCl concentrations tested, the glycine betaine content was greater than that of the other osmoprotectants.

Induction of PL production by osmolarity and its reversion by osmoprotectants

Total PL production was assayed in medium containing 0–0.7 M NaCl in the absence or the presence of 1 mM glycine betaine, proline, ectoine or pipecolate. In the absence of provided osmoprotectant, PL production first increased with increasing medium osmolarity, reaching a maximum at 0.2 M NaCl, and then decreased progressively to the basal level at 0.4 M NaCl (Fig. 2). All the osmoprotectants tested reversed the increase in PL production that was induced by NaCl. This reversion was not complete with 1 mM proline but, in the presence of the other osmoprotectants, PL production was not significantly affected by increasing NaCl concentration.

To determine whether the PL induction observed with NaCl was a reflection of osmotic strength rather than being NaCl-specific, we tested PL production in medium of similar osmotic strength using 0.3 M NaCl, 0.3 M KCl, 0.48 M mannitol or 0.41 M glycerol as osmotic agents. PL production increased from 0.04 U in absence of added osmolyte to 0.27, 0.41 and 0.17 U in the presence of NaCl, KCl or mannitol, respectively. The addition of glycerol did not modify the specific activity of PL. The different osmoprotectants reversed PL induction in the presence of KCl or mannitol, but had no effect on PL production by cells grown in glycerol-containing medium (data not shown). Thus, activation of PL production resulted mainly from osmotic pressure. Additional ionic effects could not be excluded since better induction was observed with salts than with mannitol. This effect was not related to growth rate since this latter was unaffected by NaCl levels that were effective for PL induction. With regard to PL production under osmotic stress, strain 3937 behaved quite differently from the other *E. chrysanthemi* strains tested. Mildenhall *et al.* (1981) and Mildenhall & Prior (1983) showed that only salts, and not organic solutes, were able to modify PL activity. With strain 3937, we did not observe a reduction in extracellular PL activity at any NaCl content as was reported by Prior *et al.* (1994). However, the effect observed by Prior *et al.* (1994) was mainly at the level of PL secretion; in our study, we always tested the total level and not only the extracellular fraction. The effect of osmoprotectants on PL activity that we observed also conflicts with the results of Prior *et al.* (1994), since we observed a reduction in PL activity instead of an increase. However, different isolates of *E. chrysanthemi* were used and some differences could result from strain specificity. Mildenhall *et al.* (1981) and Mildenhall & Prior (1983) reported that the nature of the medium and the osmolytes used may profoundly influence the results. They concluded that the effect of the water activity adjuster is not limited solely to water removal. It

seems possible that both water activity and osmolarity can influence PL production. Nevertheless, we propose that the increase in PL production in strain 3937 does not result from the reduction of water activity but is stimulated by the osmotic pressure, since glycerol, which does not modify the intracellular pressure, had no effect on PL production. In this context, the effect of osmoprotectants could be explained by the recovery of cellular turgor resulting from their accumulation.

***In vitro* effect of osmolytes and osmoprotectants on PL activity**

The effect of osmolarity on PL activity was analysed *in vitro* after incubation of crude extracts with 0.3 M NaCl, 0.3 M KCl, 0.48 M mannitol or 0.41 M glycerol for 30 min prior to enzyme assay. Elevated osmolyte concentrations can not be used directly in the assay medium because they provoke precipitation of the PL substrate, polygalacturonate. PL activity decreased by about 30% after incubation in the presence of NaCl or KCl. In contrast, the presence of mannitol or glycerol enhanced PL activity, by about 20%. High salt concentration probably affects the stability of the PL proteins while organic solutes protect the enzymes. The influence of 1 mM osmoprotectants, glycine betaine, proline, ectoine or pipicolate, added alone or in the presence of osmolytes, was analysed. No significant effect on PL activity was observed. Thus, osmoprotectants at low concentrations do not affect the activity of PL excreted into the growth medium.

The effect of osmolytes on *in vitro* enzymic activity may be due to an effect on one or more PL isoenzymes. A specific inhibition by salts was observed, but both mannitol and glycerol slightly enhanced PL activity. Most of the enzymes that are sensitive to medium osmolarity (Pollard & Wyn Jones, 1979; Arakawa & Timasheff, 1985; Gouesbet *et al.*, 1993) are inhibited by ionic strength rather than by water activity. The *in vitro* reversion of the salt effect was tested with low concentrations of osmoprotectants. Since PLs are secreted by *E. chrysanthemi* into the medium, they may be naturally exposed to low concentrations of these compounds but not to the high concentrations found in the intracellular compartment. Thus, there is no biological significance in testing higher concentrations of osmoprotectants such as those used for intracellular enzymes (Arakawa & Timasheff, 1985; Gouesbet *et al.*, 1993). We conclude that osmoprotectant effects on PL activity are exerted only at the level of enzyme production.

Effects of osmolarity and osmoprotectants on *pelE* expression

It was previously reported that among the *pel* genes, only *pelE* transcription was induced by the presence of 0.3 M NaCl in growth medium (Hugouvieux-Cotte-Pattat *et al.*, 1992). We tested the influence of 0–0.7 M NaCl on *pelC::uidA* and *pelE::uidA* transcriptional fusion activities. The expression of *pelC::uidA* was not affected

Fig. 3. Influence of medium osmolarity and osmoprotectants on the expression of the *pelE::uidA* fusion. Cells were grown in M63 medium containing NaCl at the indicated concentration and deprived of (▲) or containing 1 mM of glycine betaine (○), proline (●), ectoine (□) or pipicolate (■). After 10 generations in the same medium, cells were collected in the late exponential-phase and β -glucuronidase, reflecting the expression of the fusion, was assayed. Specific activity is expressed as nmol *p*-nitrophenol liberated min⁻¹ (mg dry wt)⁻¹.

by increasing the medium osmolarity up to 0.7 M NaCl, since the specific activity of β -glucuronidase was 150 U for every NaCl concentration. Similarly, the presence of 1 mM glycine betaine, proline, ectoine or pipicolate had no influence on *pelC::uidA* expression (data not shown). In contrast, *pelE::uidA* transcription was induced by NaCl; β -glucuronidase activity was maximal at 0.3 M NaCl and then decreased slowly up to 0.7 M NaCl (Fig. 3). The presence of 1 mM osmoprotectants in the growth medium reversed the induction by NaCl (Fig. 3).

As observed for PL production, the induction of *pelE::uidA* could be achieved by osmolytes other than NaCl. The addition of KCl or mannitol into the medium at a concentration corresponding to 825–866 mosM, resulted in the induction of *pelE::uidA* expression (Table 1). As for PL, β -glucuronidase specific activity was greater for cells grown with NaCl or KCl than for cells issued from mannitol-containing medium. When medium osmo-

Table 1. Influence of NaCl, KCl, mannitol and glycerol on *pelE*:*uidA* expression

Cells were grown for 10 generations in the same M63 medium containing 0.3 M NaCl (825 mosM), 0.3 M KCl (875 mosM), 0.5 M mannitol (866 mosM) or 0.5 M glycerol (1002 mosM). Specific activity is expressed as nmol *p*-nitrophenol liberated min⁻¹ (mg dry wt)⁻¹.

Osmoprotectant (1 mM)	Specific activity of β -glucuronidase with:			
	NaCl	KCl	Mannitol	Glycerol
None	1360	1070	790	50
Glycine betaine	190	120	70	50
Proline	250	150	120	60
Ectoine	220	140	80	60
Pipecolate	200	160	90	60

larity was increased with the permeant molecule glycerol, no variation in β -glucuronidase activity was observed. The effects of the various osmolytes was reversed by osmoprotectants, as was the case for NaCl-induced expression. Thus, reversion is not specific to the osmolyte, but probably results from the recovery of intracellular turgor pressure induced by osmoprotectant accumulation. We could exclude the fact that osmoprotectants act independently of turgor since they have no effect when present in medium deprived of osmolyte, or when added to medium containing 0.5 M glycerol.

Since total PL production follows this regulatory pattern, it is tempting to speculate that increase in PL activity could be attributed only to the isoenzyme PLe. Various PL isoenzymes are produced in all *E. chrysanthemi* strains. The divergence of our results with those of Prior *et al.* (1994) could result from a different proportion of PL isoenzymes in the *E. chrysanthemi* strains used. We observed, as described for formate dehydrogenase in *E. coli* (Gouesbet *et al.*, 1993), a shift between the response of gene expression and enzyme production as osmolarity increased. This result implies that, as for formate dehydrogenase synthesis in *E. coli*, osmoregulation of PL production in *E. chrysanthemi* is exerted both at the transcriptional and post-transcriptional level. As reported by Prior *et al.* (1994), PL secretion is affected by medium osmolarity. Other steps in PL maturation or folding may also be influenced by osmolarity.

Higher induction of PL activity was obtained with KCl, but NaCl was more efficient at inducing *pelE* expression. In most bacteria, KCl induces a lower osmotic response than NaCl, probably because K⁺ accumulation could partially reverse the stress by increasing free cytoplasmic water (Cayley *et al.*, 1992). The greater PL activity in the presence of KCl could be attributed to an effect of K⁺ on the secretion of PL, or its maturation or folding in the periplasmic space (Shevchik *et al.*, 1994). The osmo-induction of *pelE* could be stimulated by K⁺ as described

for *E. coli* genes (Ramirez *et al.*, 1989; Booth, 1993). Since osmoprotectants are accumulated within the cell, their effect on *pelE* expression could be attributed to an efflux of K⁺, as observed previously in *E. coli* (Cayley *et al.*, 1992; Gouesbet *et al.*, 1993). *pelE* expression was also shown to be stimulated by anaerobiosis (Hugouvieux-Cotte-Pattat *et al.*, 1992), so DNA supercoiling may also be involved in its regulation, as is the case for other genes stimulated both by osmolarity and by anaerobiosis (Dorman *et al.*, 1988; Gouesbet *et al.*, 1993).

ACKNOWLEDGEMENTS

We thank Valerie James for correcting the English in this paper, and Guy Condemine, Sylvie Reverchon and Théophile Bernard for their interest in this work. This work was supported by grants from the Centre National de la Recherche Scientifique, the Ministère de la Recherche et de la Technologie, the Direction de la Recherche et des Etudes Doctorales and the Région Bretagne (programme BRITTA).

REFERENCES

- Arakawa, T. & Timasheff, S. N. (1985). The stabilization of proteins by osmolytes. *Biophys J* **47**, 411–414.
- Bernard, T., Jebbar, M., Rassouli, Y., Himdi-Kabbab, S., Hamelin, J. & Blanco, C. (1993). Ectoine accumulation and osmotic regulation in *Brevibacterium linens*. *J Gen Microbiol* **139**, 129–136.
- Booth, I. R. (1993). Regulation of gene expression during osmoregulation: the role of potassium glutamate as a secondary signal of osmotic stress. In *Alkali Cation Transport Systems in Prokaryotes*, vol. 2, pp. 309–331. Edited by E. P. Bakker. Boca Raton, FL: CRC Press.
- Cayley, S., Lewis, B. A. & Record, M. T. (1992). Origins of the osmoprotective properties of betaine and proline in *Escherichia coli* K-12. *J Bacteriol* **174**, 1586–1595.
- Colhoun, J. (1973). Effects of environmental factors on plant disease. *Annu Rev Phytopathol* **11**, 343–364.
- Csonka, L. N. (1989). Physiological and genetic responses of bacteria to osmotic stress. *Microbiol Rev* **53**, 121–147.
- Csonka, L. N. & Hanson, A. D. (1991). Prokaryotic osmoregulation: genetics and physiology. *Annu Rev Microbiol* **45**, 569–606.
- Dorman, C. J., Barr, G. C., Ni Bhriain, N. & Higgins, C. F. (1988). DNA supercoiling and the anaerobic and growth phase regulation of *tonB* gene expression. *J Bacteriol* **170**, 2816–2826.
- Gouesbet, G., Blanco, C., Hamelin, J. & Bernard, T. (1992). Osmotic adjustment in *Brevibacterium ammoniagenes*: pipecolic acid accumulation at elevated osmolalities. *J Gen Microbiol* **138**, 959–965.
- Gouesbet, G., Abaibou, H., Wu, L. F., Mandrand-Berthelot, M. A. & Blanco, C. (1993). Osmotic repression of anaerobic metabolic systems in *Escherichia coli*. *J Bacteriol* **175**, 214–221.
- Hoppe, P. E. & Kelman, A. (1969). Bacterial top and stalk rot disease of corn in Wisconsin. *Plant Dis Rep* **53**, 66–70.
- Hugouvieux-Cotte-Pattat, N., Dominguez, H. & Robert-Baudouy, H. (1992). Environmental conditions affect transcription of the pectinase genes of *Erwinia chrysanthemi* 3937. *J Bacteriol* **174**, 7807–7818.

- Ikuta, S., Matuura, K., Imamura, S., Misaki, H. & Horiuti, Y. (1977).** Oxidative pathway of choline to betaine in the soluble fraction prepared from *Arthrobacter globiformis*. *J Biochem* **82**, 157–163.
- Jebbar, M., Talibart, R., Gloux, K., Bernard, T. & Blanco, C. (1992).** Osmoprotection of *Escherichia coli* by ectoine: uptake and accumulation characteristics. *J Bacteriol* **174**, 5027–5035.
- Kotoujansky, A. (1987).** Molecular genetics of pathogenesis by soft-rot *Erwinia*. *Annu Rev Phytopathol* **25**, 405–430.
- Mildenhall, J. P. & Prior, B. A. (1983).** Water relations of *Erwinia chrysanthemi*: intracellular and extracellular pectate lyase production. *J Gen Microbiol* **129**, 3019–3025.
- Mildenhall, J. P., Prior, B. A. & Trollope, L. A. (1981).** Water relations of *Erwinia chrysanthemi*: growth and extracellular pectic acid lyase production. *J Gen Microbiol* **127**, 27–34.
- Miller, J. H. (1972).** *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Pollard, A. & Wyn Jones, R. G. (1979).** Enzyme activities in concentrated solutions of glycine betaine and other solutes. *Planta* **144**, 291–298.
- Prior, B. A., Hewitt, E., Brandt, E. V., Clarke, A. & Mildenhall, J. P. (1994).** Growth, pectate lyase production and solute accumulation by *Erwinia chrysanthemi* under osmotic stress: effect of osmoprotectants. *J Appl Bacteriol* **77**, 433–439.
- Ramirez, R. M., Prince, W. S., Bremer, E. & Villarejo, M. (1989).** *In vitro* reconstitution of osmoregulated expression of *proU* of *Escherichia coli*. *Proc Natl Acad Sci USA* **86**, 1153–1157.
- Shevchik, V. E., Condemine, G. & Robert-Baudouy, J. (1994).** Characterization of DsbC, a periplasmic protein of *Erwinia chrysanthemi* and *Escherichia coli* with disulfide isomerase activity. *EMBO J* **13**, 2007–2012.
- Talibart, R., Jebbar, M., Gouesbet, G., Himdi-Kabbab, S., Wroblewski, H., Blanco, C. & Bernard, T. (1994).** Osmoadaptation in rhizobia: ectoine-induced salt tolerance. *J Bacteriol* **176**, 5210–5217.
-

Received 28 October 1994; revised 31 January 1995; accepted 22 February 1995.